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(54) Title: METHODS AND COMPOSITION FOR DIAGNOSING AND TREATING *PSEUDOXANTHOMA ELASTICUM* AND RELATED CONDITIONS

(57) Abstract: Methods and compositions are provided for diagnosing and treating *Pseudoxanthoma elasticum* (PXE) patinet and PXE carriers. Methods and compositions are based on the discovery that PXE mutations are located in the MRP6 (ABCC6) gene.

METHODS AND COMPOSITION FOR DIAGNOSING AND TREATING PSEUDOXANTHOMA ELASTICUM AND RELATED CONDITIONS

GOVERNMENT SUPPORT

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RELATED APPLICATIONS

This application claims priority to, and the benefit of U.S.S.N. 60/184,269, filed February 23, 2000, the disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates generally to the field of physiological dysfunctions associated with *Pseudoxanthoma elasticum*. More particularly, the invention is concerned with the identification of a gene associated with *Pseudoxanthoma elasticum*, as well as mutations in the gene that cause the disease. The present invention also relates to methods for detecting and diagnosing *Pseudoxanthoma elasticum*, to methods for identifying carriers of mutant and normal alleles of the gene associated with *Pseudoxanthoma elasticum*, to methods for screening compounds to identify potential therapeutics for *Pseudoxanthoma elasticum*, to treatment methods for *Pseudoxanthoma elasticum*, and to useful cell lines and animal models of the disease.

BACKGROUND OF THE INVENTION

Pseudoxanthoma elasticum (PXE) is a heritable disorder characterized by mineralization of elastic fibers in skin, arteries and the retina, that result in dermal lesions with associated laxity and loss of elasticity, arterial insufficiency, cardiovascular disease and retinal hemorrhages leading to macular degeneration.

The skin manifestations are among the most common characteristics of PXE, but the ocular and cardiovascular symptoms are responsible for the morbidity of the disease. Characteristic skin lesions are generally an early sign of PXE and were first described by a French dermatologist in 1896. Skin lesions are usually detected during childhood or adolescence and progress slowly and often unpredictably. Therefore, the initial diagnosis of PXE is sometimes made by a dermatologist. The skin lesions consist of yellowish papules and plaques and laxity with loss of elasticity, and result from an accumulation of abnormal mineralized elastic fibers in the mid-dermis. Lesions are typically seen on the face, neck, axilla, antecubital fossa, popliteal fossa, groin and periumbilical areas. A PXE diagnosis can be confirmed by a skin biopsy that shows calcification of fragmented elastic fibers in the mid- and lower dermis.

Another characteristic of PXE is the presence of ocular lesions due to the accumulation of abnormal elastic fibers in the Bruch' s membrane, resulting in angioid streaks. Doyne was the first to describe these ocular streaks in 1889, and Knapp introduced the term "angioid streaks" for their resemblance to blood vessels. The combination of PXE and ocular manifestations was initially referred to as the Gronblad-Strandberg syndrome, after the names of two ophthalmologists who independently related the occurrence of angioid streaks to PXE in 1929. The majority of PXE patients (approximately 85%) develop ocular manifestations during their second decade of life. Bilateral angioid streaks are normally seen as linear gray or dark red lines with irregular serrated edges lying beneath normal retinal blood vessels and represent breaks in the Bruch's membrane. The Bruch's membrane is not in a true sense a "membrane" but rather a heterogeneous elastin-rich layer separating the chorioid from the retina. The elastic laminae of the Bruch's membrane is located between two layers of collagen (type I, III and IV) which lie in direct contact with the basement membranes of the retinal pigmented epithelium (RPE) and the capillaries in the choriocapillary layer of the chorioid. As a consequence of angioid streaks, a PXE patient progressively develops a chorioidal neovascularization with a subsequent hemorrhagic detachment of the fovea and later scarring. Optic nerve drusen may also be associated with angioid streaks and results in visual field deficits and even advanced visual impairment.

Common cardiovascular complications of PXE are due to the presence of abnormal calcified elastic fibers in the internal elastic lamina of medium-sized arteries. The broad spectrum of phenotypes includes premature atherosclerotic changes, intimal fibroplasia causing

angina or intermittent claudication or both, early myocardial infarction and hypertension. Fibrous thickening of the endocardium and atrioventricular valves can also result in restrictive cardiomyopathy. Approximately 10% of PXE patients also develop gastrointestinal bleeding and central nervous system complications (such as stroke and dementia) as a consequence of systemic arterial wall mineralization. In addition, renovascular hypertension and atrial septal aneurysm can be seen in PXE patients.

Strikingly, lung abnormalities are not a significant phenotypic feature of PXE, even though pulmonary tissues are rich in elastic fibers. Mineralization of pulmonary elastic fibers has only been noted in a few patients.

PXE is usually found as a sporadic disorder but examples of both autosomal recessive and autosomal dominant forms of PXE have been reported. Partial manifestations of the PXE phenotype have also been described in presumed carriers in PXE families. Recent reports have linked both the dominant and recessive forms of PXE to a 5 cM domain on chromosome 16P13.1 However, the mechanisms underlying the physiological defects characteristic of PXE are not understood.

Therefore, there is a need in the art for methods and compositions for diagnosing and treating PXE and PXE associated phenotypes.

SUMMARY OF THE INVENTION

The invention provides methods and compositions for diagnosing and treating PXE and PXE associated physiological dysfunctions. According to the invention, mutations associated with PXE are located in the (MRP6) ABCC6 gene. Therefore, methods for detecting the presence of a mutation associated with PXE involve interrogating the (MRP6) ABCC6 gene, or a portion thereof, for the presence of one or more mutations that are associated with PXE. Accordingly, one aspect of the invention provides methods for identifying individuals that have one or two mutant alleles at the PXE locus. PXE is most often an autosomal recessive disease. Therefore, an individual with two mutant (MRP6) ABCC6 alleles associated with PXE will develop symptoms characteristic of the disease. In contrast, an individual with one mutant (MRP6) ABCC6 allele associated with PXE is a carrier of the disease and does not develop full-blown PXE. However, according to one embodiment of the invention, a PXE carrier may

develop mild forms of the characteristic manifestations. Accordingly, a PXE carrier status can be indicative of a predisposition to PXE related symptoms such as eye, skin, or cardiovascular problems. In a preferred embodiment of the invention, genetic counseling is provided to an individual identified as having a mutation associated with PXE in one or both alleles of the PXE ((MRP6) ABCC6) locus.

In another aspect, the invention provides compositions for detecting the presence of a mutation associated with PXE at the (MRP6) ABCC6 locus. In a preferred embodiment, an oligonucleotide that hybridizes to the (MRP6) ABCC6 locus is used in a diagnostic assay. In a more preferred embodiment, the oligonucleotide includes a sequence complementary to a mutation that is associated with PXE. Alternatively, an antibody-based diagnostic assay is used to detect the presence of a mutation associated with PXE at the (MRP6) ABCC6 locus.

Other aspects of the invention include therapeutic uses of the (MRP6) ABCC6 gene or protein, drug screening, the identification of (MRP6) ABCC6 homologues in other organisms (including mammalian organisms), cellular and animal models of PXE, the identification of (MRP6) ABCC6 functional domains related to the PXE phenotype, the identification of regulators of (MRP6) ABCC6 expression (mutations in these regulators can also result in PXE related symptoms), the identification of genes/proteins that interact with (MRP6) ABCC6 (alterations in these interacting molecules can also cause PXE related symptoms).

Thus, in one series of embodiments the invention provides methods for screening for the presence of a PXE mutation by interrogating an MRP6 nucleic acid obtained from a patient for the presence of a PXE mutation. The screen is positive is the presence of a PXE associated mutation is detected. A PXE associated mutation is a mutation that causes the PXE phenotype in an individual that is homozygous for the mutation. PXE associated mutations also causes the PXE phenotype in an individual that is a compound heterozygote with two different mutant alleles at the MRP6 locus, wherein each allele is a PXE associated allele. Nucleic acid is isolated from a patient biological sample, and the biological sample is preferably blood, saliva, amniotic fluid, or tissue such as a biopsy tissue. According to the invention, an MRP6 nucleic acid is a nucleic acid obtained from the MRP6 locus. An MRP6 nucleic acid can be mRNA, genomic DNA or cDNA from the MRP6 locus, or a PCR product of any of the above. According to the invention, the MRP6 locus includes the MRP6 exons, introns, and associated promoter and regulatory sequences in the genome surrounding the MRP6 exons.

In one series of embodiments, a PXE associated mutation is detected in MRP6 using a nucleic acid based detection assay. Preferred nucleic acid based detection assays include hybridization assays, primer extension assays, SSCP, DGGE, RFLP, LCR, DHPLC, and enzymatic cleavage assays. In another series of embodiments, a PXE associated mutation is detected in a protein based detection assay. Preferred protein based detection assays include ELISA and a Western blot assays. In one embodiment of the invention, mutation detection assays are provided to screen the MRP6 locus or a portion thereof to determine whether a mutation is present. The lack of MRP6 expression or the expression of a physically aberrant form of MRP6 may be sufficient to determine that an individual has a PXE associated mutation at the MRP6 locus. Alternatively, the determination that a mutation is present in the MRP6 locus may not be sufficient to determine the PXE status of an individual in the absence of information concerning the specific identity of the mutation. If such a mutation is present, it may be identified according to methods of the invention, for example by sequencing the region of the MRP6 locus that contains the mutation. Once a mutation is identified in a patient sample, the PXE status of the patient can be determined according to methods of the invention. In an alternative embodiment of the invention, specific mutation detection assays are provided to detect a known PXE associated MRP6 mutation in a patient sample.

In another series of embodiments, the invention provides oligonucleotide probes or primers and antibodies for use in mutation detection assays or screens according to the invention.

In another series of embodiments, the invention provides methods for screening candidate drug compounds to identify therapeutic compounds for treating PXE patients (individuals that have PXE due to the presence of two recessive PXE associated MRP6 alleles, or one apparently dominant PXE allele) or PXE carriers (individuals with one normal MRP6 allele and one allele with a PXE associated mutation).

In another series of embodiments, the invention provides methods for treating PXE patients or carriers using a normal MRP6 nucleic acid or protein to restore normal MRP6 function to the individual or to specific cells or tissues or the individual.

In another series of embodiments, the invention provides methods for creating transgenic or knockout cell lines and animals in order to provide a model system for PXE.

In another series of embodiments, the invention provides methods for identifying compounds such as other intracellular proteins that interact with MRP6 thereby to identify additional therapeutic targets for PXE treatment.

Accordingly, the invention provides methods and compositions for unambiguously determining the PXE status of an individual. The invention provides methods for detecting deletions, substitutions, insertions, and rearrangements in the MRP6 locus that are associated with PXE. In preferred embodiments, the invention provides methods for identifying mutations known to be associated with PXE. Preferred mutations include mutations that affect one or more of the bases in codons 1114, 1138, 1141, 1298, 1302, 1303, 1314, 1321 and other codons identified herein as being important for normal MRP6 function. Alternatively, the invention provides methods to identify mutations that result in non-conservative substitutions in the MRP6 locus. In a further embodiment, the invention provides assays to detect PXE associated mutations at intron/exon splice sites of the MRP6 gene. The invention also provides methods to detect mutations that affect one or more regulatory elements of the MRP6 gene, including the promoter, the polyA site and other transcriptional or translational control sequences.

Methods of the invention are also useful to screen a population in order to identify individuals with one or more PXE associated MRP6 alleles. According to the invention, these individuals are provided with appropriate genetic counseling in view of their PXE status.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of the MRP6 gene and the surrounding genomic regions. Two (MRP6) *ABCC6* mutations that cause PXE are indicated.

Figure 2 shows the predicted topology of the MRP6 protein and the location of ten mutations causing PXE.

Figure 3 shows conserved amino acids in the human MRP6 protein.

Figure 4 shows co-segregation of the PXE phenotype with the R1141X mutation in exon 24 of the (MRP6) *ABCC6* gene.

Figure 5 shows segregation of the PPXE phenotype for an apparent autosomal dominant mutation.

Figure 6 shows a construct for deleting exon 28 in a mouse.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods and compositions for diagnosing and treating PXE and PXE related symptoms. Methods and compositions of the invention rely in part on the discovery that mutations associated with PXE map to the (MRP6) ABCC6 gene locus on chromosome 16. Accordingly, the invention provides useful PXE related diagnostic and therapeutic methods and compositions by exploiting wild-type and mutant (MRP6) ABCC6 genes and proteins.

I. PXE Associated Mutations in the (MRP6) ABCC6 gene

a) Mapping of PXE associated mutations to the (MRP6) ABCC6 genetic locus

Although the first case of PXE was reported by Darier in 1896, most PXE cases have been reported since the 1970s. In most reports, PXE is inherited as an autosomal recessive (AR) phenotype or appears as a sporadic phenotype. However, kindreds showing apparent autosomal dominant (AD) inheritance have also been reported. Using DNA from patients and unaffected family members from 21 unrelated PXE families, the PXE phenotype was linked to the short arm of chromosome 16. A very significant linkage with an 8 cM region was demonstrated with a maximum lod score of 8.07. A subsequent haplotype analysis and recombination mapping reduced the locus from 8 cM to 820 kb where six candidate genes were identified. The locus was later reduced to less than 600 kb and one candidate gene was excluded. All 109 exons of the five remaining candidate genes were screened by a combination of single-strand conformation polymorphism (SSCP), heteroduplex analysis (HA) or direct sequencing using genomic DNA from a cohort of 17 unrelated PXE patients and three unrelated normal individuals. The first six mutations, clearly associated with the PXE phenotype, were found in the (MRP6) ABCC6 gene (also known as the ABCC-6 gene). This analysis is described in further detail in Example 2. According to the invention, the MRP6 gene has 31 exons as shown in Figure 1. A 107.7 kb genomic sequence that includes the MRP6 locus is shown in SEQ ID NO: 1. The sequence of SEQ ID NO: 1 shows the complementary strand of the MRP6 gene. The intron/exon boundaries are as follows (on the complementary strand of SEQ ID NO: 1): Ex1: 102783-102748; Ex2:

101180-100998; Ex3: 99296-99171; Ex4: 99031-98903; Ex5: 93798-93676; Ex6: 91594-91533; Ex7: 88207-88076; Ex8: 82954-82757; Ex9: 81524-81347; Ex10: 77528-77367; Ex11: 72268-72176; Ex12: 69718-69515; Ex13: 68325-68182; Ex14: 66562-66475; Ex15: 64385-64310; Ex16: 62282-62156; Ex17: 61940-61764; Ex18: 58324-58157; Ex19: 56985-56811; Ex20: 55345-55270; Ex21: 52757-52637; Ex22: 49588-49381; Ex23: 45578-45268; Ex24: 42837-42638; Ex25: 41209-41083; Ex26: 39226-39125; Ex27: 37453-37307; Ex28: 34674-34516; Ex29: 34437-34271; Ex30: 30412-30218; Ex31: 29881-29773. The mRNA coding sequence for human MRP6 is shown in SEQ ID NO: 2, and the encoded protein sequence is shown in SEQ ID NO: 3.

b) Identifying PXE associated mutations in the (MRP6) ABCC6 locus

According to methods of the invention, additional PXE associated mutations were identified in the (MRP6) ABCC6 locus using a combination of single strand conformation polymorphism (SSCP), heteroduplex analysis (HA) and direct sequencing. Single nucleotide mutations in the (MRP6) ABCC6 gene were identified in several cohorts of individuals originating from the United States, South Africa and several European countries (Belgium, Germany, Holland, Italy and United Kingdom). To confirm the causative or polymorphic nature of new variants, a control panel of 300 alleles (150 normal individuals) was screened and the co-segregation of the identified variant and the PXE phenotype was verified. It is noteworthy that two single-allele mutations (Rll4lX, R1339C) were found in control panels of normal individuals indicating that heterozygote mutant (MRP6) *ABCC6* alleles can be found in the normal population. However, the missense mutation (Rl339C) was identified in the genetically distinct Afrikaners of South Africa. The frequency of heterozygote carriers deduced only from the appearance of these heterozygote mutations is 1.3 percent and is consistent with the commonly accepted figures of 0.6 to 2.5%. Indeed, while most mutations appeared to be private, a few have been clearly identified as recurrent (Rll4lX, R518Q, 3775delT, 16.5 kb deletion between exon 22 and 29. Most of the mutations (63%) were missense substitutions, 17% were nonsense mutations (5), 13% were frameshift mutations (4 deletions or an insertions of a single nucleotide) and 7% were likely to affect splicing (2).

Twenty-seven of the mutations (90%) affected the C-terminal half of the (MRP6) ABCC6 protein and particularly the various domains of the C-terminal ATP-binding site, which are encoded by exons 28 to 30, where 12 (40%) mutations were clustered. Remarkably, 10 mutations (33%) affected arginyl residues. Eight of these were missense substitutions, suggesting an essential structural or functional role for these arginyl residues in (MRP6) ABCC6.

Large deletions, which are not detected by SSCP or HA, can be identified by the loss of heterozygosity of informative polymorphic markers. Seven highly informative microsatellites present in a 300 kb region encompassing both *ABCC1* and *ABCC6*, have been successfully used to detect large deletions involving parts or the entire *ABCC6* gene. The loss of heterozygosity can also be efficiently implemented by using several highly polymorphic variants present in the *ABCC6* gene. The latter approach was used to detect a partial deletion of the (MRP6) *ABCC6* gene, in a compound heterozygous state, in a family with an apparent dominant form of PXE, as discussed in Example 3. A non-limiting list of known PXE associated mutations at the MRP6 locus are shown in Table 1.

Mutations		Status		Exons
Effect	**Nt change**	**Status**	**Origin**	
-	938-939insT	ch,ht	A	8
R518Q	1553G>A	ch,ht	a, u	12
F568S	1703T>C	ht	U	13
L673P	2018T>C	ch	A	16
-	1995delG	ch	G	16
-	2322delC	ht	U	18
Y768X	2204C>A	ch, ht	A	18
-	IVS21+1G>T	ch	U	Intron 21
R1030X	3088C>T	ht	A	23
R1114P	3341G>C	hm	Uk	24
S1121W	3362C>G	ch	G	24
R1138P	3413G>C	ch	G	24
R1138Q	3413G>A	ch	Uk	24

Mutations		Status		Exons
Effect	Nt change	Status	Origin	
R1141X	3421C>T	all	All	24
G1203D	3608G>A			25
-	IVS26-1G>A	ch	B	Intron 26
W1241C	3723G>C			26
Q1237X	3709C>T	ch	B	26
-	3775delT	ht, hm	a, u, h	27
V1298F	3892G>T	ht	U	28
T1301I	3902C>T	ch	B	28
G1302R	3904G>A	ht	U	28
A1303P	3907G>C	ch	B	28
R1314W	3940C>T	hm	U	28
R1314Q	3941G>A	ht	G	28
G1321S	3961G>A	ht	U	28
R1339C	4015C>T	all	a, u	28
Q1347H	4041G>C	ht	U	28
D1361N	4081G>A	ch	G	29
R1398X	4192C>T	ch	B	29
I1424T	4271T>C	ht	U	30

ch= compound heterozygote; ht= heterozygote; hm= homozygote; ivs= intervening sequence

Table 1. Known PXE associated mutations at the human MRP6 locus.

According to methods of the invention, additional PXE associated mutations can be identified in the (MRP6) ABCC6 locus according to methods of the invention. For example, single strand conformation polymorphism (SSCP), heteroduplex analysis (HA), or direct sequence analysis can be used to identify additional mutations at the MRP6 locus. In one embodiment, the analysis is performed on genomic DNA. Alternatively, the analysis is performed on cDNA or on exon containing DNA amplification products such as exon containing PCR products. Deletion mutations are preferably detected using diagnostic PCR assays of genomic DNA and by Southern hybridization according to

methods known in the art. In addition, fluorescent in situ hybridization (FISH) analysis of human chromosome preparations can be used to identify a deletion at the MRP6 locus or a deletion that encompasses all or part of the MRP6 locus. Specific mutations are preferably identified using DNA arrays including mutation specific oligonucleotide probes. Alternatively, mutation-specific antibodies can be used to detect mutations that alter an existing epitope or create a new specific epitope on the MRP6 protein. Preferably, specific antibodies are used on proteomic chips to detect protein altering mutations in the MRP6 gene. Mutations can also be detected using mass spectrometry, and mutation-specific mass spectrometer profiles can be generated for MRP6 nucleic acid or protein analysis according to methods known in the art.

c) PXE Associated Mutations at the (MRP6) ABCC6 Locus

i) The (MRP6) ABCC6 gene and protein

The (MRP6) ABCC6 gene, also known as the ABBC6 gene, encodes an ATP-binding cassette transporter (an ABC transporter) belonging to sub-family "C" which includes genes involved in drug-resistance such as MRP1 to 6 (ABCC1-6). (MRP6) ABCC6/ABCC6 encodes a 165 kDa protein that is located in the plasma membrane and has 17 membrane-spanning helices grouped into three transmembrane domains. MRP6 is highly homologous to MRP1 and may act as an efflux pump of amphipathic anion conjugates. Accordingly, in one aspect of the invention, MRP6 transports glutathione anion conjugates and also anionic drugs. Therefore, an individual that is a PXE carrier or a PXE homozygote or compound heterozygote may have reduced transport of anionic drugs and may be more receptive to chemotherapy using such drugs. The ABCC family of genes also includes the cystic fibrosis transmembrane conductance regulator gene *(ABCC7 or CFTR)* and the sulfonylurea receptor genes *(ABCC8 and* 9 *or SUR).*

Therefore, in contrast to genetic changes involved in other elastic fiber diseases such as Supravalvular Aortic Stenosis (SVAS), Marfan syndrome, and *Cutis laxa,* PXE associated mutations in the (MRP6) ABCC6 gene are not directly related to elastic fibers. The (MRP6) ABCC6/ABCC6 gene is expressed at relatively high levels in a limited range of tissues, notably in kidney and liver. However, low levels of expression are also observed in smooth muscle cells and macrophages. According to the invention, this

tissue distribution suggests that (MRP6) ABCC6 has a function related to cellular detoxification which may affect the calcification of elastic fibers in skin, arteries and the retina. Alternatively, calcification of elastic fibers in skin, arteries, and the retina may result from MRP6 functional deficiencies in those tissues.

The predicted structure of the MRP6 protein is shown in Fig. 2. Transmembrane domains (unshaded rectangles), nucleotide-binding fold regions (NBF) and Walker motifs are indicated and were identified by amino acid sequence homology with similar transporters. Arrows indicate the positions of several PXE associated mutations. The large shaded rectangle represents the cell membrane.

According to the invention, the transmembrane domains of the MRP6 protein shown in Fig. 2 are hydrophobic stretches of amino acids identified via transmembrane domain predictions (SOSUI and DAS transmembrane prediction programs, http://www.biokemi.su.se/-server/DAS/; http://azusa.proteome.bio.tuat.ac.jp/sosui/). Regions of MRP6/ABCC6 with a high degree of conservation when compared with similar proteins (ABC transporters) include the regions involved in the binding and hydrolysis of ATP also known as nucleotide binding folds (NBF). According to the invention, the MRP6 protein has two nucleotide-binding fold regions (NBF1 and NBF2) as shown in Fig. 2. These regions correspond to the following amino acid segments of the human MRP6 protein: NBF1 residues 656-679, 747-768, and 775-784 of SEQ ID NO: 3; and NBF2 residues 1292-1307, 1321-1327, and 1403-1433 of SEQ ID NO: 3.

According to one embodiment of the invention, conserved amino acids in the MRP6 protein are amino acids identified by comparing 12 ABC transporter proteins from Human, Rat, Mouse, C. elegans, Yeast (S. cerevisiae) and A. thaliana. Preferred conserved amino acids are shown in Fig. 3 (conserved amino acids are underlined). According to the invention, conserved domains are concentrated in the C-terminal portion of the protein, where over 90% of the PXE causing mutations have been identified.

ii) Mutations in the (MRP6) ABCC6 gene

According to one aspect of the invention, PXE is caused by a mutation at the (MRP6) ABCC6 locus that results in reduced MRP6 protein function. PXE associated mutations include mutations that affect the level of MRP6 protein expression in addition

to mutations that alter the functional properties of an expressed MRP6 protein. PXE associated mutations at the (MRP6) ABCC6 locus include chain-terminating mutations. Such mutations are typically recessive and account for the autosomal recessive nature of the associated PXE phenotype. However, PXE associated mutations identified at the (MRP6) ABCC6 locus include chain terminating mutations at different positions in the (MRP6) ABCC6 gene, and several substitution, deletion and insertion mutations. According to the invention, the C-terminal half of the MRP6 protein is functionally important. Indeed, many of the PXE associated mutations were identified in exons 23-29. However, even a I to T substitution at position 1424 (out of 1503 amino acid residues) results in a PXE associated phenotype. Accordingly, a chain terminating or frameshift mutation in any one of exons 1-29, even up to position 1424 in exon 30, and maybe even beyond is expected to be associated with PXE. According to the invention, the PXE phenotype associated with different mutations in the (MRP6) ABCC6 gene varies in relation to the functional properties of the mutant (MRP6) ABCC6 protein product. Therefore, individuals with different PXE associated mutations can have PXE symptoms of differing severity. In addition, different individuals having the same PXE mutations, but in different genetic backgrounds, can also develop PXE symptoms of differing severity. Accordingly, different mutations at the PXE locus are expected to result in PXE phenotypes of differing severity. For example, in one embodiment of the invention, a mutation that results in the absence of MRP6 protein expression (for example a deletion of part or all of the gene, a chain terminating mutation, a mutation that prevents mRNA production, or a mutation that prevents translation of the mRNA) is expected to have a more severe PXE phenotype than a mutation that interferes with normal MRP6 protein function without destroying the function (for example an amino acid substitution that alters the structure and function of the protein without inactivating it. In particular, an individual that is a homozygote for a mutation that prevents MRP6 protein expression, or that is a compound heterozygote with two different mutations each of which prevents MRP6 protein expression, is expected to have a more severe phenotype than an individual that has a mutation with less severe effects on MRP6 protein function at one or both alleles of the MRP6 locus.

In a further embodiment of the invention, a heterozygote carrier of a PXE mutation can exhibit characteristic manifestations of PXE. In particular, a carrier of a recessive mutation can show partial skin, eye or cardiovascular symptoms. According to the invention, heterozygote carriers of different (MRP6) ABCC6 mutations can develop different subsets of PXE related symptoms and can have symptoms of varying severity. Indeed, there are numerous examples of dermal "elastic fibers changes" or cardiovascular abnormalities ranging from hypertension to myocardial infarction, in family members of severely affected individuals. According to the invention, cases of partial expression of PXE symptoms in heterozygote carriers are cases that had been assumed to be examples of dominant inheritance with for example 10 to 20% penetrance.

The various subtypes of a disorder or a dual mode of inheritance of a disease are frequently due either to mutations in different genes or different mutations in the same gene. Epidermolysis buflosa (EB) is an excellent example of a disorder characterized by several clinical types caused by distinct mutations in the same gene or mutations in different genes. EB is viewed as a group of heritable mechano-bullous skin diseases classified into three major categories of simplex, junctional and dystrophic forms. EB simplex is due to mutations in the genes encoding keratins 5 and 14, the junctional form is associated with mutations in the *kalininl7aminin 5* genes; and the dystrophic disorder result from mutations in the type VII collagen gene *(COL7Al)*. The dystrophic EB presents clinical sub-types: the Hallopeau-Siemens type is autosomal recessive and caused by nonsense mutations and glycine substitutions result in the autosomal dominant form.

In contrast to EB, no locus heterogeneity has been shown for PXE. According to the invention, most cases of PXE, if not all, are due to (MRP6) ABCC6 mutations. While the clinical heterogeneity in PXE patients may be caused by different types of (MRP6) ABCC6 mutations, the different PXE lesions (vascular, ocular, and dermal) observed for different autosomal recessive and seemingly dominant PXE mutations are clinically indistinguishable. Furthermore, identical PXE mutations can be either recessive or apparently dominant in unrelated pedigrees. Accordingly, different PXE mutations in different genetic backgrounds are associated with different severities of PXE symptoms.

Furthermore, a PXE mutation can result in a partial PXE phenotype in a carrier individual (thereby accounting for observations of apparent dominant forms of PXE).

iii) Population Distributions of (MRP6) ABCC6 mutations

According to the invention, different PXE associated (MRP6) ABCC6 mutations exist in the population, and new (MRP6) ABCC6 mutations arise sporadically. Based on current estimations of the prevalence of PXE in the United States (between 1:100,000 and 1:25,000), the frequency of appearance of heterozygote individuals with PXE mutations should be between 0.6 and 2.5 percent of the general population (1.5 to 6.0 million individuals). Given the risk of heterozygote individuals having children with PXE, an important aspect of the invention is to provide a genetic screen to identify heterozygote carriers of PXE mutations. According to the invention, a PXE carrier is an individual with one mutant allele of the (MRP6) ABCC6 gene, wherein the mutant allele is an allele that results in a PXE phenotype in an individual that is homozygous for that allele (or in an individual that is heterozygous with two different (MRP6) ABCC6 mutant alleles, each of which is associated with PXE).

According to a further embodiment of the invention, a significant factor in the complex phenotype of the PXE multi-organ disorder is partial expression of the full range of the PXE symptoms in heterozygote carriers in recessive pedigrees. For example, a single mutant-ABCC6 allele, for example R1141X, within heterozygote carriers can manifest a partial, mostly vascular-related phenotype. Indeed, cardiovascular abnormalities are frequently seen in obligate carriers but ocular and dermal lesions have also been diagnosed. The PXE phenotype, as observed in several heterozygous carriers, range from sub-clinical manifestations to visible lesions. The spectrum of these partial phenotypes overlaps with that of the less severely affected PXE patients. There is, therefore, a continuum in the PXE phenotype between heterozygous carriers and PXE patients, which make the clinical diagnosis of the less severe forms of PXE equivocal. According to the invention, cardiovascular symptoms associated with PXE mutations at the MRP6 gene include atherosclerosis, hypertension, stroke, gastrointestinal bleeding, intermittent claudication. Ocular symptoms include macular or retinal degeneration and skin related symptoms include premature aging and solar elastosis.

According to the invention, the identification of the PXE gene provides methods for an unambiguous molecular diagnosis of patients and the identification of heterozygous carriers in families with autosomal recessive PXE or apparent autosomal dominant PXE, and the identification of homozygous PXE individuals or PXE carriers in the general population.

According to the invention, different populations can contain different characteristic PXE associated MRP6 mutations or different frequencies of PXE associated MRP6 mutations due to factors such as founder effects. For example, a founder effect in the South African Afrikaner population is thought to have caused the observed higher frequency of PXE in Afrikaners. According to the invention, a higher frequency of PXE in a population correlates with a higher frequency of PXE associated MRP6 mutations.

Intra-familial variation of the phenotype is a well known characteristic of *PXE.* These variations may be due to genetic and/or environmental causes. A few environmental factors are thought to influence the PXE phenotype. Among these, calcium and Vitamin D have been reported to contribute to the severity of the phenotype in some cases. Life style, smoking, diet, sun-exposure and obesity are also likely to modulate the penetrance of the phenotype. Indeed, remarkably dissimilar PXE phenotypes have been observed recently in identical twins. According to the invention, non-genetic factors contributing to the development of PXE symptoms in heterozygote carriers can be identified. Studies involving large cohorts of twins for example, such as those used by the Queensland Institute of Medical Research of Australia (http://genel2i.qimr.ed are also useful to identify both genetic and environmental factors related to the development of the PXE phenotype.

II. Diagnostic Applications

(MRP6) ABCC6 genes and gene products, including mutant genes and gene products, as well as probes, primers, and antibodies, are useful for identifying carriers of PXE associated mutations. According to the invention, PXE associated mutations can be identified in families with a PXE pedigree or in individuals not previously known to be at risk of carrying a PXE related mutation. PXE associated mutations can be routinely

screened using probes to detect the presence of a mutant (MRP6) ABCC6 gene or protein by a variety of methods. In preferred embodiments of the invention, individuals are screened for the presence of a recurrent mutation that is known to be present at a relatively high frequency in the population. For example, a preferred method of the invention screens an individual from a population for the presence of an MRP6 mutation that accounts for about 30%, and preferably 50%, and more preferably over 50%, of known incidences of PXE in the population. An alternative method of the invention screens an individual for the presence of two or more, preferably about five, more preferably about ten, and even more preferably over ten PXE associated MRP6 mutations. In methods that include assays for a plurality of PXE associated MRP6 mutations, the plurality of mutations preferably account for about 30%, and more preferably 50%, and even more preferably over 50%, of known incidences of PXE in the population.

In one aspect of the invention, the identification of a specific mutation is not necessary. A diagnostic assay may be based on the detection of an MRP6 protein expression defect resulting from, for example, reduced levels of mRNA expression. Indeed, the analysis of steady state levels of (MRP6) *ABCC6* mRNA in skin fibroblasts from a PXE patient carrying a homozygous R1141X mutation showed that MRP6 mRNA levels were lower than in skin fibroblasts from a normal individual. Accordingly, low levels MRP6 mNRA can result from a mutation within the coding sequence, such as a nonsense mutation that results in nonsense mediated decay. In addition, low mRNA levels can be caused by mutations either an intron or an exon that destabilizes the RNA, or by a mutation in a regulatory region (including a promoter region) that reduces transcription of the MRP6 gene. Furthermore, the presence of a truncated MRP6 mRNA can be used as a diagnostic indicator for the presence of a PXE associated mutation.

Alternatively, the presence of a mutation that affects the amount, size, or other physical properties of the MRP6 protein can be detected without knowing the identity of the mutation. For example a decreased level of MRP6 protein or a the presence of a truncation in the MRP6 protein can be used as a diagnostic indicator for the presence of a PXE associated mutation. In addition, the presence of a larger than expected MRP6 protein (that may result for example, from a gene fusion or from one or more frameshift

mutations that produce a larger and possibly non-functional protein) can be used as a diagnostic inidicator for the presence of a PXE associated mutation.

Accordingly the invention provides a method for screening for the presence of a PXE associated mutation at the MRP6 locus without specifically identifying the mutation. Such methods are useful to identify homozyogtes, compound heterozygotes, or carriers.

According to the invention, the identification of the presence of any PXE associated mutation at the MRP6 locus can be used as a positive diagnosis of PXE in an individual with PXE symptoms or to diagnose a PXE patient who has not yet developed PXE symptoms but who is identified as a homozygote or a compound heterozygote for PXE associated MRP6 mutations. Alternatively, the detection of the presence of a PXE associated MRP6 mutation according to the invention provides a method for screening a population to identify individuals who are carriers of a PXE associated mutation.

In general, a PXE carrier is distinguished from a PXE homozygote by the presence of both a normal allele and a PXE mutant allele in the carrier and the presence of two PXE mutant alleles in the homozygote. According to the invention, a normal allele can contain a neutral polymorphism as disclosed herein.

a) Nucleic acid based diagnostics

When a diagnostic assay is to be based upon nucleic acids from a sample, the assay may be based upon mRNA, cDNA or genomic DNA. If mRNA is used from a sample, there may be little or no expression of transcripts unless appropriate tissue sources are chosen or available. Preferred tissue sources are biopsies of full thickness skin or skin fibroblasts cultured from dermal biopsies. Whether mRNA, cDNA or genomic DNA is assayed, standard methods well known in the art may be used to detect the presence of a particular sequence either in situ or in vitro (see, e.g., Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY). As a general matter, however, any tissue with nucleated cells may be examined.

Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The

DNA may be isolated and used directly for detection of a specific sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification. To detect a specific nucleic acid sequence, direct nucleotide sequencing, hybridization using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNase protection, chemical mismatch cleavage, ligase-mediated detection, and various other methods may be employed. Oligonucleotides specific to particular sequences can be chemically synthesized and labeled radioactively or non-radioactively (e.g., biotin tags, ethidium bromide), and hybridized to individual samples immobilized on membranes or other solid-supports (e.g., by dot-blot or transfer from gels after electrophoresis), or in solution. The presence or absence of the target sequences may then be visualized using methods such as autoradiography, fluorometry, or colorimetry. These procedures can be automated using redundant, short oligonucleotides of known sequence fixed in high density to silicon chips, or in other oligonucleotide array formats.

Whether for hybridization, RNase protection, ligase-mediated detection, PCR amplification or any other standards methods described herein and well known in the art, a variety of subsequences of the MRP6 sequences disclosed or otherwise enabled herein will be useful as probes and/or primers. These sequences or subsequences will include both normal MRP6 sequences and PXE associated MRP6 mutant sequences. In general, useful oligonucleotide probes or primer sequences will include at least 8-9, more preferably 10-50, and most preferably 18-24 consecutive nucleotides from the MRP6 introns, exons or intron/exon boundaries. Depending upon the target sequence, the specificity required, and future technological developments, shorter sequences may also have utility. Therefore, any MRP6 derived sequence which is employed in a diagnostic assay may be regarded as an appropriate probe or primer. Particularly useful sequences include nucleotide positions from the MRP6 gene for which PXE associated mutations are known, or sequences which flank these positions.

As discussed above, a variety of PXE causing mutations have now been identified at the human MRP6 gene locus. Detection of these and other PXE associated mutations is now enabled using isolated nucleic acid probes or primers derived from normal or mutant MRP6 genes. According to the invention, useful oligonucleotide probes or

primers are derived from sequences encoding the C-terminal half of the MRP6 protein, the conserved NBF sequences, and conserved amino acid sequence shown in Fig. 3. Particularly useful oligonucleotides are derived from sequences known to have PXE associated mutations, such as the sequences including the mutations shown in Table 1. As disclosed above, a number of PXE associated MRP6 mutations have already identified, and it is expected that more will be identified according to the compositions and methods disclosed herein. Therefore, the present invention provides isolated nucleic acid probes and primers corresponding to normal and mutant sequences from any portion of the MRP6 gene, including exons, introns, and 5' and 3' UTRs, which may be shown to be associated with the development of PXE.

Merely as an example, and without limiting the invention, useful diagnostic probes and primers derived from the MRP6 DNA are disclosed in Example 5.

For in situ hybridization-based detection of a normal or mutant MRP6, a sample of tissue may be prepared by standard techniques and then contacted with one or more of the nucleic acids described herein, preferably one which is labeled to facilitate detection, and an assay for nucleic acid hybridization is conducted under stringent conditions which permit hybridization only between the probe and highly or perfectly complementary sequences. For the single nucleotide substitutions associated with PXE, high stringency hybridization conditions will be required to distinguish most mutant sequences from normal sequences. When the MRP6 genotypes of an individual's parents are known, probes may be chosen accordingly. Alternatively, probes to a variety of mutants may be employed sequentially or in combination. Because PXE carriers will be heterozygous, probes to normal sequences also may be employed and homozygous normal individuals may be distinguished from mutant heterozygotes by the amount of binding (e.g., by intensity of radioactive signal). In another variation, competitive binding assays may be employed in which both normal and mutant probes are used but only one is labeled.

In addition to oligonucleotide-based hybridization assays, methods of the invention include direct sequencing, loss of heterozygosity, SSCP, HA, and Conformation-Sensitive Gel Electrophoresis (CSGE) to detect a PXE associated MRP6 mutation. As discussed above, preferred mutations to be screened for are those shown in

Table 1. However, additional mutations identified according to the invention are also useful as markers of PXE, including deletions in the (MRP6) ABCC6 locus.

According to one embodiment of the invention, a diagnostic test can be a nucleic scanning test where the assay detects the presence of a mutation in the nucleic acid being interogated. In an alternative embodiment, a diagnostic test can interrogate a nucleic acid for the presence of a specific mutation.

According to this invention, base pair deletions or alterations leading to the omission of amino acid residues in the gene product are determined. Nucleic acid primers and probes are used in a variety of PCR-based amplification and hybridization assays to screen for and detect the presence of defective ABCC6 gene or mRNA in a patient. The genetic information derived from the intron/exon boundaries is also very useful in various screening and diagnosis procedures.

Various nucleic acid scanning methods are used for scanning the MRP6 genomic, mRNA or cDNA sequence obtained from a patient for detecting, for example, large deletions and substitutions in the sequence that would be indicative of the disease. These nucleic acid scanning techniques include PCR-based techniques and using oligonucleotide probes that hybridize to specific regions of the gene.

In one embodiment of the invention, preferred mutations for nucleic acid scanning techniques include large deletions in the genomic sequence for the ABCC6 gene for example a 16.5 kb deletion spanning from exon 22 to exon 29. Primers are designed to various regions of the ABCC6 gene which are used for PCR-based detection of large deletions in the gene.

In another embodiment of the invention, primers are designed to the ABCC6 gene that are able to differentiate between the size of the amplified wild-type sequence and sequence containing a specific mutation, for example a deletion.

In a preferred embodiment of the invention, nucleic acid probes are provided which comprise either ribonucleic or deoxyribonucleic acids. Typically, the size of the probes varies from approximately 18 to 22 nucleotides. Functionally, the probe is long enough to bind specifically to the homologous region of the ABCC6 gene, but short enough such that a difference of one nucleotide between the probe and the DNA being

tested disrupts hybridization. Thus the nucleic acid probes of the present invention are capable of detecting single nucleotide changes in the ABCC6 gene.

In a preferred embodiment of the invention, nucleic acid probes are 100 % homologous to a mutant allele of the ABCC6 gene, but not to the wild-type gene.

In another embodiment of the invention, the nucleic acid probes are 100 % homologous to the wild-type allele. Accordingly, the invention provides methods for determining whether an individual is homozygous or heterozygous for a particular allele using both a wild-type and an allele-specific probe.

According to one method of the invention, mutations are detected by sequencing specific regions of the ABCC6 gene. In a preferred embodiment, the specific regions encompass one or more mutations presented in Table 1. In an alternative embodiment, a specific region being interrogated includes one of exons 1-31. Preferred exons include exons 25-29, and more preferably exon 28 in which many PXE associated mutations have been identified.

According to still other methods of the present invention rapid screening techniques are used to determine whether exons of the ABCC6 gene carry any mutations. Such techniques can be followed by one of the techniques already described above which are specific for a particular allele or mutation. One such rapid screening technique involves the determination of the conformation of single strands of DNA which have been amplified from exon sequences that are known to carry mutations, including the mutations presented in Table 1. The single strands are run in non-denaturing electrophoretic gels, such as are typically used for sequencing DNA. The mobility of single stranded DNA on such gels is sensitive to the conformation of the DNA fragments. The conformation of the single stranded DNA is dependent on its base sequence, alterations in even one base affecting the conformation. Thus the presence of a wild-type or mutant allele described herein can be detected by amplifying an exon sequence, denaturing the duplex molecules, and separating them on the basis of their conformation on non-denaturing polyacrylamide gels. If mutant alleles are present, they will have a different mobility than wild-type sequences amplified with the same primers. Most conveniently, the amplified sequences will be radiolabeled to facilitate visualization on gels. This can be readily accomplished using labeled primers or a labeled nucleotide. For

a general reference on this technique see Orira, et al., Genomics vol. 5, pp. 874-879 (1989). A preferred nucleic acid amplification product for SSCP analysis is between about 100 and 500 bp, and more preferably between about 140 and 300 bp.

According to another rapid screening technique of the present invention, an amplified fragment containing a mutation is detected using denaturing gradient gel electrophoresis (DGGE). For a general reference on this technique see Sheffield, et al., Proc. Natl. Acad. Sci. vol. 86, pp. 232-236 (1989). Briefly, double stranded fragments which are generated by amplification (PCR) can be subjected to DGGE. "DGGE is a gel system that separates DNA fragments according to their melting properties. When a DNA fragment is electrophoresed through a linearly increasing gradient of denaturants, the fragment remains double stranded until it reaches the concentration of denaturants equivalent to a melting temperature (Tm) that causes the lower-temperature melting domains of the fragment to melt. At this point, the branching of the molecule caused by partial melting sharply decreases the mobility of the fragment in the gel. The lower-temperature melting domains of DNA fragments differing by as little as a single-base substitution will melt at slightly different denaturant concentrations because of differences in stacking interactions between adjacent bases in each DNA strand. These differences in melting cause two DNA fragments to begin slowing down at different levels in the gel, resulting in their separation from each other." Sheffield, et al., ibid. Use of a GC clamp as taught in Myers et al., Nucleic Acids Res. vol. 13, pp. 3111-3146 (1985) increases the sensitivity of detection of this method from about 40% to about 100%. If mismatches are present, which would be the case if the DNA sample amplified was heterozygous for an ABCC6 allele, they will be visible on these DGGE gels. Double stranded fragments containing one wild-type strand and one mutant strand will have a different mobility on these gels than will double stranded fragments which contain two wild-type or two mutant strands, due to the different melting temperatures of these species. Thus, the melting temperature of fragments amplified from different regions of the ABCC6 gene can be determined by DGGE and can be used to indicate whether a mutant allele is present.

In one embodiment, a region of the (MRP6) ABCC6 gene that encodes an important functional domain of the (MRP6) ABCC6 protein is screened for the presence

of any mutation. For example, a preferred diagnostic assay interrogates the region of the (MRP6) ABCC6 gene that encodes an ATP binding site of the (MRP6) ABCC6 protein, a region that encodes a hydrophobic transmembrane domain, or a region that encodes a conserved amino acid, preferably in the C-terminal half of the MRP6 protein.

One major application of the nucleic acid based diagnostics is in the area of genetic testing, carrier detection and prenatal diagnosis. Individuals carrying mutations in the ABCC6 gene (disease carrier or patients) may be detected at the DNA level with the use of a variety of techniques. The genomic DNA used for the diagnosis may be used directly for detecting specific sequences or may be amplified enzymatically in vitro, for example by PCR. The detection of specific DNA sequence may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al. Cold Spring Harbour Symp. Quant. Biol. 51: 257-261 (1986)), direct DNA sequencing (Church and Gilbert, Proc. Nat. Acad. Sci. U. S. A. 81: 1991-1995 (1988)), the use of restriction enzymes (Flavell et al. Cell 15: 25 (1978), Geever et al Proc. Nat. Acad. Sci. U. S. A. 78: 5081 (1981)), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis, Cold Spring Harbour Sym. Quant. Biol. 51: 275-284 (1986)), RNase protection (Myers, R. M., Larin, J., and T. Maniatis Science 230: 1242 (1985)), chemical cleavage (Cotton et al Proc. Nat. Acad. Sci. U. S. A. 85: 4397-4401, (1985)) and the ligase-mediated detection procedure (Landegren et al Science 241:1077 (1988)).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labelled radioactively with isotopes or non-radioactively (with tags such as biotin (Ward and Langer et al. Proc. Nat. Acad. Sci. U. S. A. 78: 6633-6657 (1981)), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these specific sequences are visualized by methods such as autoradiography or fluorometric (Landegren et al, 1989) or colorimetric reactions (Gebeyshu et al. Nucleic Acids Research 15: 4513-4534 (1987)).

Sequence differences between normal and mutants may be revealed by the direct DNA sequencing method of Church and Gilbert. Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly

enhanced when combined with PCR (Wrichnik et al, Nucleic Acids Res. 15:529-542 (1987); Wong et al, Nature 330:384-386 (1987); Stoflet et al, Science 239:491-494 (1988)). In the latter procedure, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. For example, a PCR product with a small deletion is clearly distinguishable from the normal sequence on an 8% non-denaturing polyacrylamide gel. DNA fragments of different sequence compositions may be distinguished on denaturing formamide gradient gel in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperature (Myers, supra). In addition, sequence alterations, in particular small deletions, may be detected as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis, as have been detected for the 3 dp (I507) mutation and in other experimental systems (Nagamine et al, Am. J. Hum. Genet, 45:337-339 (1989)). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length in a PCR. For example, one invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.

Sequence alterations may occasionally generate fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization (Southern, J. Mol. Biol 98: 503 (1975)). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme;

fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

In another embodiment of the invention, sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase (Myers, supra) and S1 protection (Berk, A. J., and P. A. Sharpe Proc. Nat. Acad. Sci. U. S. A. 75: 1274 (1978)), the chemical cleavage method (Cotton, supra) or the ligase-mediated detection procedure (Landegren supra).

In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments may also be visualized by methods where the individual DNA samples are not immobilized on membranes. The probe and target sequences may be both in solution or the probe sequence may be immobilized (Saiki et al, Proc. Natl. Acad. Sci USA, 86:6230-6234 (1989)). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving colorigenic reactions and fluorometry involving fluorogenic reactions, may be used to identify specific individual genotypes.

In a preferred embodiment of the invention, for example, a PCR with multiple, specific oligonucleotide primers and hybridization probes, may be used to identify a plurality of possible mutations at the same time (Chamberlain et al. Nucleic Acids Research 16: 1141-1155 (1988)). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki et al, supra).

According to the invention, assays are performed to detect a deletion within or including the MRP6 gene using Southern hybridization, FISH analysis, or diagnostic PCR. It is expected that most deletions will occur between repetitive Alu sequences that are common within the introns of the MRP6 gene. Preferred PCR primers for detecting these deletions are primers that flank intron Alu sequences.

According to one aspect of the invention, many of the PXE associated MRP6 mutations are found in exons 22-30. Accordingly, preferred assays of the invention interrogate any one of exons 22-30, taken alone or in combination, for the presence of a PXE associated MRP6 mutation.

In a preferred embodiment of the invention, a diagnostic assay interrogates the entire (MRP6) ABCC6 locus for the presence of a mutation, using for example SSCP, HA,

or CSGE, and direct sequencing. In a more preferred embodiment, an assay interrogates a portion of the ABCC6 locus for the presence of a mutation. If a mutation is detected, it is first compared to known mutations associated with PXE (Table 1) and known neutral polymorphisms (Table 2) that are not associated with PXE. If the mutation has not yet been observed as either a PXE associated mutation or as a neutral polymorphism, the nature of the mutation is considered. If the mutation is a deletion, nonsense, frameshift or other mutation that affects expression of a normal MRP6 protein, the mutation is considered to be a PXE mutation. Similarly, if the mutation results in a nonconservative amino acid change or an amino acid change in a conserved sequence such as an NBF, a transmembrane sequence, or a change in a conserved amino acid shown in Fig. 3, the mutation is considered to be a PXE mutation. In addition, if the mutation results in low levels of MRP6 expression, the mutation is considered to be a PXE mutation. However, if the mutation results in a conservative amino acid change in a non-conserved part of the MRP6 protein the mutation is considered to be a neutral polymorphism. Nonetheless, a patient identified with a previously unknown neutral polymorphism according to this analysis should be subjected to additional diagnostic tests to look for known PXE associated symptoms or subclinical symptoms.

According to one aspect of the invention, the detection of PXE carriers and PXE patients is determined through the identification of mutant MRP6 alleles in DNA from patients, family members and apparently unrelated and normal individuals. A single allele, with no evidence of a second mutant allele and the presence of a normal allele will be considered a carrier. Patients may be identified as either compound heterozygotes (having two different mutant alleles) or homozygotes (two identical mutant alleles).

b) Protein based diagnostics

Different approaches to a MRP6 protein-based diagnostic assay can be used to detect the presence of a PXE related mutation in a patient. Preferred assays include detecting a mutant electrophoretic mobility, the presence of a mutant epitope, the absence of a normal epitope, or by identifying altered biological activity, for example altered ATP binding or altered transport of a synthetic, preferably radiolabeled molecule.

In one embodiment, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

In preferred embodiments, protein-based diagnostics will employ differences in the ability of antibodies to bind to normal and mutant MRP6 proteins. Such diagnostic tests may employ antibodies which bind to the normal proteins but not to mutant proteins, or vice versa. In particular, an assay in which a plurality of monoclonal antibodies, each capable of binding to a mutant epitope, may be employed. The levels of anti-mutant antibody binding in a sample obtained from a test subject (visualized by, for example, radiolabelling, ELISA or chemiluminescence) may be compared to the levels of binding to a control sample. Alternatively, antibodies which bind to normal but not to mutant MRP6 protein may be employed, and decreases in the level of antibody binding may be used to distinguish homozygous normal individuals from mutant heterozygotes or homozygotes. Such antibody diagnostics may be used for in situ immunohistochemistry using biopsy samples of tissues obtained from patients.

c) Genetic counseling

According to one embodiment of the invention, genetic counseling is provided to an individual identified as a PXE carrier, a PXE homozygote, or a PXE compound heterozygote (an individual with two different PXE mutant alleles). According to the invention, individuals carrying two PXE mutant alleles are provided information about ameliorating treatments for some of the symptoms of PXE. For example, a person who inherits PXE recessively is cautioned with regard to diet and activity. A low fat, high fibre, heart healthy diet is critical for maintaining cardiovascular health. Regular exercise appears to alleviate some of the symptoms of peripheral vascular disease. Medications to allow the passage of blood through narrowed arteries may be recommended. Individuals exhibiting eye manifestations should not engage in activities that put them at risk for

injury to the eye that could subsequently lead to hemorrhage and vision loss. Smoking should be avoided at all costs since it appears to increase the rate and severity of eye disease. In one embodiment of the invention, a patient identified as being a carrier of a PXE associated mutation or as being a homozygote or a compound heterozygote for PXE associated mutations should be advised to reduce calcium intake or to use drugs that reduce calcium intake in order to reduce the severity of the phenotype.

III. Therapeutic applications

The present invention provides a basis for therapeutic treatments of PXE related symptoms caused by mutations at the (MRP6) ABCC6 locus. According to the invention, normal (MRP6) ABCC6 nucleic acid or protein is provided to cells and/or a patient having a PXE associated mutation at the (MRP6) ABCC6 locus.

Preferred target tissues include the kidney and liver, but also other tissues where low levels of MRP6 expression have been observed, such as smooth muscle cells and macrophages. Preferred target tissues also include tissues or cells that exhibit PXE related symptoms, such as a blood vessel, the gastrointestinal tract, occular tissue, the urinary tract, and skin.

a) Nucleic acid-based therapeutics

According to the invention, PXE or PXE associated symptoms can be prevented or treated by providing a normal PXE gene or cDNA to a patient that is diagnosed as having on or more PXE associated mutations at the (MRP6) ABCC6 locus. The fact that PXE is a recessive disease makes it particularly amenable to gene therapy, because it is expected that most, if not all, PXE associated MRP6 mutations reduce the amount of functional MRP6 protein in a cell and can be compensated for by providing normal MRP6 to the cell.

In one series of embodiments, normal copies of the MRP6 gene are introduced into patients to code successfully for normal protein in one or more different affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Thus, it is preferred that the recombinant gene be operably joined to a strong promoter so as to provide a high level of

expression which will compensate for the absence of sufficient amounts of normal MRP6. As noted above, the recombinant construct may contain endogenous or exogenous regulatory elements, inducible or repressible regulatory elements, or tissue-specific regulatory elements.

Preferred vectors for introducing an MRP6 gene to a cell or a patient include retroviral vectors, because of their high efficiency of infection and stable integration and expression. Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus such as Epstein-Barr virus. Alternative vectors include plasmids that are replicated in human cells.

In another series of embodiments, a mutant MRP6 gene may be replaced by homologous recombination with a recombinant construct. The recombinant construct preferably contains a normal copy of the MRP6 gene. Alternatively, a regulatory region of a normal MRP6 gene in a PXE carrier may be altered to increase expression of normal MRP6.

i) Wild type genes

In one series of embodiments, a normal human (MRP6) ABCC6 gene is introduced to cells or a patient. A normal (MRP6) ABCC6 gene includes a gene with one or more polymorphic variations that are not associated with PXE. In one embodiment, an MRP6 genomic sequence is used. In an alternative embodiment an MRP6 cDNA sequence is used.

ii) Related genes

In an alternative series of embodiments, an (MRP6) ABCC6 related gene is provided to a cell or tissue having a PXE associated mutation. According to the invention, an (MRP6) ABCC6 related gene encodes a protein that has similar functional properties as a normal human MRP6 protein and can compensate for the absence of sufficient amounts of normal human MRP6 protein in a patient cell or tissue. Preferably, an (MRP6) ABCC6 homologue from another mammalian species is used. For example the mouse or rat MRP6 genes or cDNAs could be used. In one embodiment of the invention,

a homologue from a non-mammalian species is used. Alternatively, a nucleic acid encoding a different ABC protein is used, for example an MRP1 encoding nucleic acid.

The present invention also provides for cells or cell lines, both prokaryotic and eukaryotic, which have been transformed or transfected with the nucleic acids of the present invention so as to cause clonal propagation of those nucleic acids and/or expression of the proteins or peptides encoded thereby. Such cells or cell lines will have utility both in the propagation and production of the nucleic acids and proteins of the present invention but also, as further described herein, as model systems for diagnostic and therapeutic assays. As used herein, the term "transformed cell" is intended to embrace any cell, or the descendant of any cell, into which has been introduced any of the nucleic acids of the invention, whether by transformation, transfection, infection, or other means. Methods of producing appropriate vectors, transforming cells with those vectors, and identifying transformants are well known in the art.

b) Protein based therapeutics

Treatment of PXE symptoms may be performed by directly providing normal protein to a patient cell or tissue. Sufficient amounts of substantially pure MRP6 protein can be obtained from cultured cell systems which express the protein. Delivery of the protein to the affected tissue can then be accomplished using appropriate packaging or administrating systems including, for example, liposome mediated protein delivery to the target cells.

c) Drug Therapies

In one embodiment of the invention, a drug identified according to methods of the invention is administered to a patient diagnosed with PXE or a PXE carrier with PXE related symptoms . Alternatively, a drug is administered to prevent or minimize the development of PXE or PXE associated symptoms in individuals identified as having one or more PXE mutations at the ABCC6 locus.

IV. Drug Discovery applications

The present invention provides a basis for screening drug candidates to identify useful therapeutic compositions to treat or alleviate the symptoms of PXE. In a series of embodiments, the invention provides screens based on MRP6 activity. As used with respect to this series of embodiments, the term "activity" broadly includes gene and protein expression, protein post-translation processing, trafficking and localization, and any functional activity (e.g., enzymatic, receptor-effector, binding, channel), as well as downstream effects of any of these. MRP6 appears to be an integral membrane protein and may have transport related functions, and it also has ATP binding cassettes. Accordingly, these functional properties can be used as a basis for a screen to identify compounds that increase MRP6 function.

In one embodiment, a drug candidate is screened for its ability to increase expression of the MRP6 gene. A preferred screen monitors the level of normal MRP6 mRNA in cells grown in culture in the presence and absence of the candidate compound. Alternatively, normal MRP6 protein levels are monitored. Useful cells for these assays are preferably normal cells or PXE carrier cells. However, a PXE cell can also be used and the levels of mutant MRP6 expression can also be monitored. A compound that increases the level of MRP6 expression is particularly useful to treat a PXE carrier in order to increase the level of MRP6 expressed from the normal allele. However, a compound that increases the level of MRP6 expression can also be useful to treat a PXE homozygote or compound heterozygote if the PXE associated MRP6 allele(s) encodes an MRP6 protein that retains some normal MRP6 function or if the allele is a mutation that reduces the level of normal MRP6 function.

Other assays are useful for screening candidate compounds to identify a compound that increases normal MRP6 function. In one embodiment, an assay screens a compound for the ability to restore normal phenotype to dermal fibroblasts isolated from a PXE patient. Dermal fibroblasts isolated from patients with PXE exhibit abnormal phenotype when grown in vitro (Quaglino et al., Biochimica et Biophysica Acta 1501 (2000) 51-62). These phenotypes include an increased proliferation index compared to normal fibroblasts when grown in monolayer. PXE fibroblasts also have lower adhesion properties to collagen type I and to plasma fibronectin when compared to normal

fibroblasts. Accordingly, these phenotypes provide a basis for an assay to identify a compound that restores normal MRP6 function to dermal fibroblasts isolated from a patient that was identified as having a PXE associated MRP6 mutation.

In another embodiment of the invention, an assay is used to screen candidate compounds for their ability to increase the ATPase activity of an MRP6 proteins. In a preferred embodiment, the assay monitors the ATPase activity of an MRP6 protein encoded by an MRP6 gene with a PXE associated mutation in the presence and absence of the candidate compound. ATPase activity of purified MRP6 can be assayed according to methods known in the art (see, for example, Mao et al., Biochimica et Biophysica Acta 1461, 69-82 (1999). According to the invention, a compound that increases the ATPase activity of a PXE associated MRP6 protein variant is useful to treat a patient that is heterozygous or homozygous for the PXE allele that encodes the protein variant used in the assay.

In a similar embodiment of the invention, an assay is used to screen candidate compounds for their ability to increase the transport activities of an MRP6 protein, in particular a PXE associated MRP6 protein variant. A useful transport assay is provided in Oude et al., Biochim Biophys Acta, 1241(2), 215-68, 1995. A compound identified according to this screen is useful to treat PXE patients and PXE carriers as described above.

V. Disease Models

The invention provides a basis for designing cellular and animal models of PXE. Such models are useful to study the development of the PXE disease in PXE homozygotes and compound heterozygotes and to identify potential PXE associated physiological dysfunctions in PXE carriers. Such models are also useful in screens to identify therapeutic compounds to prevent or treat PXE symptoms.

a) Cellular models

According to the invention, cellular models can be made by deleting one or both MRP6 alleles, or by introducing one or more PXE associated MRP6 alleles into a cell line grown *in vitro*, using methods known in the art. Preferred cell lines include renal and

hepatic cell lines. Other useful cell lines include those derived from skin (keratinocytes and fibroblasts) and ocular tissue (ganglioma cells).

b) Animal models

The present invention also provides for the production of transgenic non-human animal models for the study of PXE, for the screening of candidate pharmaceutical compounds, and for the evaluation of potential therapeutic interventions.

Animal species which suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates (e.g., Rhesus monkeys, chimpanzees). For initial studies, transgenic rodents (e.g., mice) are preferred due to their relative ease of maintenance and shorter life spans. Transgenic yeast or invertebrates (e.g., nematodes, insects) may be preferred for some studies because they will allow for even more rapid and inexpensive screening. Transgenic non-human primates, however, may be preferred for longer term studies due to their greater similarity to humans.

Based on the identification of MRP6 as the gene associated with PXE, there are now several available approaches for the creation of a transgenic animal models for PXE, including animal models with one or both MRP6 alleles deleted and animal models with one or two MRP6 alleles with mutations similar to known PXE associated human MRP6 mutations.

To create an animal model (e.g., a transgenic mouse), a mutant MRP6 gene can be inserted into a germ line or stem cell using standard techniques of oocyte microinjection, or transfection or microinjection into embryonic stem cells. Animals produced by these or similar processes are referred to as transgenic. If the mutation knocks out the MRP6 gene or a portion thereof, the animals are referred to as knockouts.

For oocyte injection, one or more copies of the recombinant DNA constructs of the present invention may be inserted into the pronucleus of a just-fertilized oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn animals are screened for integrants using analysis of DNA (e.g., from the tail veins of offspring mice) for the presence of the inserted recombinant transgene sequences. The transgene may be either a complete genomic sequence injected as a YAC, BAC, PAC or

other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

Retroviral infection of early embryos can also be done to insert the recombinant DNA constructs of the invention. In this method, the transgene is inserted into a retroviral vector which is used to infect embryos (e.g., mouse or non-human primate embryos) directly during the early stages of development to generate chimeras, some of which will lead to germline transmission.

Homologous recombination using stem cells allows for the screening of gene transfer cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of blastocysts, and a proportion of the resulting animals will show germline transmission from the recombinant line. In a preferred embodiment, inactivation of the MRP6 gene in mice may be accomplished by designing a DNA fragment which contains sequences from an MRP6 exon flanking a selectable marker. Homologous recombination leads to the insertion of the marker sequences in the middle of an exon, causing inactivation of the MRP6 gene and/or deletion of internal sequences. DNA analysis of individual clones can then be used to recognize the homologous recombination events.

The techniques of generating transgenic animals, as well as the techniques for homologous recombination or gene targeting, are now widely accepted and practiced. A laboratory manual on the manipulation of the mouse embryo, for example, is available detailing standard laboratory techniques for the production of transgenic mice (Hogan et al., 1986). A large number vectors are available to accomplish this and appropriate sources of genomic DNA for mouse and other animal genomes to be targeted are commercially available from companies such as GenomeSystems Inc. (St. Louis, Missouri, USA). The typical feature of these targeting vector constructs is that 2 to 4 kb of genomic DNA is ligated 5' to a selectable marker (e.g., a bacterial neomycin resistance gene under its own promoter element termed a "neomycin cassette"). A second DNA fragment from the gene of interest is then ligated downstream of the neomycin cassette but upstream of a second selectable marker (e.g., thymidine kinase). The DNA fragments are chosen such that mutant sequences can be introduced into the germ line of the

targeted animal by homologous replacement of the endogenous sequences by either one of the sequences included in the vector. Alternatively, the sequences can be chosen to cause deletion of sequences that would normally reside between the left and right arms of the vector surrounding the neomycin cassette. The former is known as a knock-in, the latter is known as a knock-out. Example 5 describes a knockout of most of exons 28 and 29 in mouse MRP6.

VI. (MRP6) ABCC6 Interacting Molecules

According to the invention, molecules that interact with a normal MRP6 gene product gene product provide candidates 1) for identifying additional types of mutations that result in a PXE phenotype and 2) for additional levels of therapeutic intervention to overcome or minimize the effect of a mutant PXE gene product. For example, the identification of a protein that interacts with a normal MRP6 protein but not with a PXE mutant protein provides a potential target for therapeutic intervention if the function of the interacting protein can be modified to compensate for the absence of normal MRP6 protein.

According to the invention, (MRP6) ABCC6 interacting molecules can be identified according to a number of biochemical and genetic methods known in the art, including affinity chromatography, mutational analysis, and yeast two hybrid analysis. As will be obvious to one of ordinary skill in the art, there are numerous other methods of screening individual proteins or other compounds, as well as large libraries of proteins or other compounds (e.g., phage display libraries and cloning systems from Stratagene, La Jolla, CA) to identify molecules which bind to normal or mutant MRP6 proteins. All of these methods comprise the step of mixing a normal or mutant MRP6 protein or protein fragment with test compounds, allowing for binding (if any), and assaying for bound complexes.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Materials and Methods

a) Sources of Patient Samples

PXE International, Inc.

To date, PXE International has assembled a database of over 2100 PXE patients from 1400 families from 31 countries including North America, several European and South American countries and South Africa. From this cohort of patients and family members, genomic DNA has been prepared from whole blood samples obtained from over 1200 PXE patients and family members.

Honolulu Heart Program

In the early 1950's, studies around the world were reporting geographic differences in coronary heart disease (CHD) mortality, pathology, prevalence, and incidence. Among these reports were those of significant differences in the CHD and cerebrovascular disease rates in Japan and in the United States. The overall mortality for men in Japan and in the United States was similar, but the rates for CHD were strikingly different. Reported CHD mortality among Japanese was approximately twenty percent of that among U.S. Caucasians. At about the same time, Japanese living in Hawaii and California were reported to have a lower overall mortality than either U.S. Caucasians or Japanese living in Japan. The reasons for these differences were not apparent. However, it was felt that the study of these populations might offer important clues to the etiology of heart and vascular disease. The compared populations, living in Japan, Hawaii and California were of Japanese ancestry to limit genetic variation between study groups. The Honolulu cohort of the Ni-Hon-San (Nippon-Honolulu, San Francisco) study formed the basis for the Honolulu Heart Program (HHP). That study has now been underway for 35 years, providing extensive information about the role of lifestyle, diet, and other risk factors to development of chronic diseases of major public health importance in 8,000 Japanese-American men.

Although several studies have suggested that PXE is more frequent in females there is no evidence that the observed gender difference is caused by genetic factors.

Indeed, men would often report skin lesions, usually the first signs of PXE, later in life, than women. Therefore, a study of an exclusively male cohort from the HHP should not compromise the general applicability of the conclusions. PXE has also no particular predilection for any ethnic or racial group. PXE has indeed largely been described in Caucasians but also in African and Asian populations. PXE cases reported in Japan have shown no phenotypic or prevalence differences when compared to those observed in Caucasian populations.

Unaffected control subjects

DNA has been prepared from 150 unrelated individuals with no evidence of PXE. These samples have been aliquoted and are currently stored at -80°C. They are routinely used as control DNA samples and will be used to confirm that any new and potential mutation detected in a PXE patient or relative is indeed a mutation and not a neutral polymorphism. The donors of these DNA samples were adults of either sex from various ethnic backgrounds.

b) Mutation Detection Methods

Detection of single nucleotide mutations

Single strand conformation polymorphism (SSCP) analysis is based on the observation that single stranded DNA will adopt, in non-denaturing conditions, a secondary structure that is strictly sequence-dependant. Slight variations in sequence, such as a single nucleotide change can alter the conformation of a DNA fragment, which can be resolved on a non-denaturing polyacrylamide gel. Heteroduplex analysis (HA) is based on the observation that heteroduplexes formed between two DNA strands with one or more mismatches have electrophoretical mobility distinctly different from homoduplexes. While both methods (SSCP and HA) can detect point mutations, some sequence variants are more readily detected by one procedure than the other. Accordingly, a preferred screening method, uses a combination of SSCP and modified HA called Conformation-Sensitive Gel Electrophoresis or CSGE.

In a preferred assay, each characterized PCR primer pair is radioactively labeled using T4 polynucleotide kinase and γ-[P^{32}]-ATP. For SSCP analysis, radiolabeled PCR products are mixed with denaturing loading buffer and loaded onto a 0.5 x MDE (MDE is a mutation detection enhancement polyacrylamide-derived matrix provided by FMC products), 0.6 x TBE native polyacrylamide gel and electrophoresed overnight at 8 watts in a sequencing gel apparatus. Separated, radiolabeled conformers are visualized by autoradiography. For CSGE, EDTA is added to the incubated PCR reaction mix to a final concentration of 1 mM and the reaction will be heat-denatured and incubated for 60 minutes at 68°C to allow heteroduplex formation. Heteroduplex products are analyzed on a 6% polyacrylamide gel (29:1 ratio of acrylamide/bisacrylamide), 10% (v/v) ethylene glycol and 15% (w/v) formamide in O.5X TTE buffer (1 X TTE is 89 mM Tris, 15 mM taurine, 0.5 mM EDTA, pH 9.0). A solution of 20% (v/v) ethylene glycol, 30% (w/v) formamide and 0.05% xylene cyanol and bromophenol blue is mixed equally with the samples. The gel is run at 35 to 45 watts for 2 to 4 hours at room temperature. As for SSCP, CSGE conformers are revealed by autoradiography.

When an abnormal conformer or heteroduplex is detected, the segregation of the variant is analyzed for DNA samples from the entire family. Subsequently, the DNA sequence of the variant is determined by eluting normal and altered DNA conformers directly from the electrophoresis gel. These PCR fragments are eluted in water, re-amplified and directly used as a template for sequencing using an ABI 310 automated sequencer (Perking Elmer). A panel of 150 DNA samples of normal unrelated individuals is used to identify abnormal variants that are common polymorphisms in the ABCC6 locus.

Mutation detection by enzymatic cleavage

Single nucleotide substitutions often modify the recognition site of a restriction enzyme. Polymorphisms and mutations can, therefore, be detected in a rapid and convenient manner by the enzymatic cleavage of a PCR fragment containing the nucleotide change. This method is frequently employed to verify the presence of previously characterized mutations or polymorphisms in DNA samples for control, study or diagnostic purposes. It can also be used for screening a large number of samples. Out

of the *ABCC6* mutations listed in Table 1, 10 mutations were identified with a unique restriction pattern. For example, three possible *HhaI* restriction profiles for one of these mutations, R1339C (4015C to T) can be visualized by electrophoresis. According to the invention, single nucleotide mutations in *ABCC6* are detectable by enzymatic cleavage. Accordingly, this method is useful as an initial step to appropriately complement the screening of large cohorts with more traditional mutations detection techniques.

PCR mapping

A single base substitution mutation may be detected based on differential PCR product length or production in PCR. Thus, primers which span mutant sites or which, preferably, have 3' termini at mutation sites, may be employed to amplify a sample of genomic DNA, mRNA or cDNA from a subject. A mismatch at a mutational site may be expected to alter the ability of the normal or mutant primers to promote the polymerase reaction and, thereby, result in product profiles which differ between normal subjects and heterozygous and/or homozygous MRP6 mutants. The PCR products of the normal and mutant gene may be differentially separated and detected by standard techniques, such as polyacrylamide or agarose gel electrophoresis and visualization with labeled probes, ethidium bromide or the like. Because of possible non-specific priming or readthrough of mutation sites, as well as the fact that most carriers of mutant alleles will be heterozygous, the power of this technique may be low.

Electrophoretic mobility

Genetic testing based on DNA sequence differences also may be achieved by detection of alterations in electrophoretic mobility of DNA, mRNA or cDNA fragments in gels. Small sequence deletions and insertions, for example, can be visualized by high resolution gel electrophoresis of single or double stranded DNA, or as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis. MRP6 mutations or polymorphisms may also be detected by methods which exploit mobility shifts due to single-stranded conformational polymorphisms (SSCP) associated with mRNA or single-stranded DNA secondary structures.

Chemical cleavage of mismatches

Mutations in MRP6 may also be detected by employing the chemical cleavage of mismatch (CCM) method. In this technique, probes (up to ~ 1 kb) may be mixed with a sample of genomic DNA, cDNA or mRNA obtained from a subject. The sample and probes are mixed and subjected to conditions which allow for heteroduplex formation (if any). Preferably, both the probe and sample nucleic acids are double-stranded, or the probe and sample may be PCR amplified together, to ensure creation of all possible mismatch heteroduplexes. Mismatched T residues are reactive to osmium tetroxide and mismatched C residues are reactive to hydroxylamine. Because each mismatched A will be accompanied by a mismatched T, and each mismatched G will be accompanied by a mismatched C, any nucleotide differences between the probe and sample (including small insertions or deletions) will lead to the formation of at least one reactive heteroduplex. After treatment with osmium tetroxide and/or hydroxylamine to modify any mismatch sites, the mixture is subjected to chemical cleavage at any modified mismatch sites by, for example, reaction with piperidine. The mixture may then be analyzed by standard techniques such as gel electrophoresis to detect cleavage products which would indicate mismatches between the probe and sample.

Other methods

Various other methods of detecting MRP6 mutations, based upon the MRP6 sequences disclosed and otherwise enabled herein, will be apparent to those of ordinary skill in the art. Any of these may be employed in accordance with the present invention. These include, but are not limited to, nuclease protection assays (S1 or ligase-mediated), ligated PCR, denaturing gradient gel electrophoresis (DGGE), restriction endonuclease fingerprinting combined with SSCP (REF-SSCP), and the like.

Methods for analyzing MRP6 mRNA levels:

The steady state levels of (MRP6) *ABCC6* mRNA was analyzed in skin fibroblasts from a PXE patient carrying a homozygous R1141X mutation. Total skin fibroblast RNA from an unaffected control individual and a PXE patient was used to synthesize single stranded cDNA using oligo(dT). PCR primers derived from (MRP6) ABCC6 mRNA sequence

were then used in two consecutive rounds of 25 cycles of PCR. Poly(A)+ RNA from normal human kidney (obtained from Clontech) was used as a positive control for detection of (MRP6) *ABCC6* mRNA. *MRP-1* mRNA was detected in the same cDNA samples used for *ABCC6* mRNA with 30 cycles of PCR. The 390 bp and 180 bp DNA fragments detected correspond to the expected size of (MRP6) *ABCC6* and *MRP-1* mRNA domains encoded within exons 6-9 and 2-3 of the (MRP6) ABCC6 and MRP-1 genes respectively. No reverse transcriptase (No RT) controls were included to confirm that no PCR products were obtained in the absence of cDNA synthesis.

Stringent hybridization conditions

High stringency conditions are at least equivalent to a temperature in the range of about 40-70 degrees C, and between about 0.05 and 0.5 M sodium ion. High stringency hybridization conditions are well known in the art and can be optimized for a specific oligonucleotide based on the length and GC content of the oligonucleotide as described in, for example, Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor, N.Y., 1982).

An oligonucleotide selected for hybridizing to the target nucleic acid, whether synthesized chemically or by recombinant DNA methodologies, is isolated and purified using standard techniques and then preferably labeled (e.g., with ^{35}S or ^{32}P) using standard labeling protocols. A sample containing the target nucleic acid then is run on an electrophoresis gel, the dispersed nucleic acids transferred to a nitrocellulose filter and the labeled oligonucleotide exposed to the filter under stringent hybridizing conditions, *e.g.* 50% formamide, 5 X SSPE, 2 X Denhardt's solution, 0.1% SDS at 42°C, as described in Sambrook *et al.* (1989) *supra*. The filter may then be washed using 2 X SSPE, 0.1% SDS at 68°C, and more preferably using 0.1 X SSPE, 0.1% SDS at 68°C. Other useful procedures known in the art include solution hybridization, and dot and slot RNA hybridization. Optionally, the amount of the target nucleic acid present in a sample is then quantitated by measuring the radioactivity of hybridized fragments, using standard procedures known in the art.

Example 2: The positional cloning of the PXE gene

Blood samples and skin biopsies from PXE patients and unaffected relatives in the United States were collected by PXE International Inc., by Dr. Ivonne Pasquali-Ronchetti in Italy, by Dr. F. Michael Pope in the United Kingdom and by Dr. Anne de Paepe in Belgium. Blood samples were obtained from 100 Caucasian control individuals with no family history of PXE. Genomic DNA was isolated from aliquots of blood. Low passage and confluent skin fibroblasts were obtained from 3 mm full thickness skin biopsies using known procedures.

To identify the gene that contains mutations responsible for PXE, the disease locus was confined to a region of about 8 cM between markers D16S500 and- D16S3041. Recombination mapping reduced this large critical region to an 820 kb domain containing six candidate genes. These genes encode an isoform of Myosin Heavy Chain (MYH11), two Multidrug Resistance-associated Proteins (MRP-1 and (MRP6) ABCC6), an unknown protein called pM5 and two identical unknown proteins referred to as UNK. Using a polymorphic microsatellite repeat *($GAAA_{17}$)* located at the 5' end of the *MRP-1* gene (Fig. 1) an informative meiotic recombination in one PXE patient was identified and this permitted the exclusion of the *MYH11* as a candidate gene and reduced the size of the PXE region to 570 kb and 5 candidate genes. Figure 1 shows the previously defined PXE locus covering 820 kb between markers D16S3060 and D16S79 at 16p13.1. The BAC contig that covers this region is shown along with the identity of the BACs. Figure 1b shows the gene content of the PXE locus represented from the telomere (left) to the centromere (right). The transcriptional orientation of the genes is indicated by arrows. A flag represents the position of a polymorphic marker ($GAAA_{17}$) used to identify an additional meiotic recombination in one PXE patient that excluded the *MYHII* gene as the PXE gene.

The 109 exons within the five candidate genes were then screened for mutations by Single-Strand Conformation Polymorphism (SSCP) and Heteroduplex Analysis (HA) using genomic DNA from a cohort of 20 unrelated PXE patients.

Mutation detection, sequence analysis and RT-PCR, SSCP, and Heteroduplex Analysis (HA) were carried out as previously described. Intron-derived primers for PCR amplification of exons present in the genes encoding MRP-1, (MRP6) ABCC6, pM5 and

both *UNK* gene were synthesized using intron sequences available in the TIGR database (http://www.tigr.org). PCR products were typically 150-350 bp in length and included complete intron/exon boundaries. Typical PCR reactions, were performed in the presence of ^{32}P-labelled primers in a 9700 thermocycler (Perkin Elmer). Radioactive PCR products were analyzed either by SSCP or HA using MDE polyacrylamide gel (FMC) according to the manufacturer's instructions. DNA conformers were eluted in water from gel slices, re-amplified and sequenced utilizing the same primers used to generate these PCR products. DNA sequence analysis was performed using ABI BigDye terminator cycle sequencing with an ABI310 automated DNA sequencer. The sequence information generated by the sequencer was analyzed using the ABI software. The Sequencher™ 3.1 program was used to identify variation between the sequence of putative mutations and control sequences. RT-PCR was performed on total RNA from cultured human skin fibroblasts and human kidney poly(A)+ RNA. The sequences of the PCR primers used are: (MRP6) *ABCC6: 5'*-AGCCACGTTCTGGTGGGTTT-3' (SEQ ID NO: 4); 5'-GGAGGCTTGGGATCACCAAT-3' (SEQ ID NO: 5); *MRP-1: 5-*CAACTGCATCGTTCTGTTTG-3' (SEQ ID NO: 6); and 5'-ATACTCCTTGAGCCTCTCCA-3' (SEQ ID NO: 7). Following synthesis, PCR products were separated by electrophoresis through 1.2% agarose and visualized by staining with ethidium bromide.

DNA sequence analysis of two conformers detected in PCR products containing exons 19 and 28 of the pM5 gene revealed two private single nucleotide polymorphisms (SNPs) within the intronic sequence flanking these exons (Table 2). These were the only sequence variants detected in the 31 exons of the pM5 gene using a cohort of 20 PXE patients. Screening all eight exons of each of the two *UNK* genes revealed only one SNP in the first exon of either one or both UNK genes in 5 PXE patients. This was a silent nucleotide change (C33T) within the 11th codon (S11) of the open reading frame of either one or both unknown genes and therefore unrelated to PXE. Screening all 31 exons of the *MRP-1* gene in a panel of PXE patients identified several sequence variants (Table 2) that are not functionally related to PXE as they either occurred in intronic sequences or did not encode changes in amino acids. In addition, two missense variants (R633Q and G671V) were seen in exons-14 and 16 in two unrelated PXE patients but these

substitutions were unlikely to be responsible for PXE as they were also found in a panel of 200 alleles from unaffected, ethnically matched control individuals.

Finally, in screening the 31 exons of the (MRP6) *ABCC6* gene, the first mutations that are clearly responsible for PXE were identified. A C->T substitution within exon 24 (C3421T) of the (MRP6) ABCC6 gene generated a stop codon at position *1141 (R1141X;* Fig. 1 and Table 2). Figure 1c shows the intron/exon structure of the (MRP6) ABCC6 gene. Intron sizes are drawn approximately to scale and the exons are numbered from the 5' end of the (MRP6) *ABCC6* gene. Figure 1d shows chromatograms of partial DNA sequence from two unrelated PXE patients containing a nonsense and a splice site mutation in exon 24 and intron 21 respectively. In exon 24, the sequence shows a 3421C>T substitution (arrowhead), which would generate a stop codon at position 1141 (R1141X). PXE patients in a consanguineous Italian pedigree were found to be homozygous for this stop codon mutation. In intron 21, a G to T substitution *(IVS21+1G>T)* was observed within the invariant GT sequence of the donor splice site. This mutation would influence constitutive splicing of (MRP6) ABCC6 pre-mRNA and was found in two unrelated PXE patients as a compound heterozygote in association with either R1141X or R1138Q. Firgure 1c shows the sequence of the normal and mutant nucleotide and amino acid sequences for the nonsense mutation in exon 24 and the splice site variant within intron 21.

The C3421T variant in exon 24, which was not found in the control panel of 200 normal alleles, co-segregated in a homozygous form with a recessive PXE phenotype in an Italian family in which all unaffected individuals but one were heterozygote carriers (Fig. 4). Figure 4 shows a large consanguineous Italian pedigree, SSCP conformers for a homozygous variant (R1141X) in exon 24 were noted in all four PXE patients (shaded symbols). All other unaffected family members were heterozygote for this nonsense mutation except one unaffected family member, indicated by an arrow. SSCP conformers from normal unrelated control DNA have been included. Total RNA from the PXE patient indicated by was used for an RT PCR analysis of (MRP6) *ABCC6* mRNA and shown to have low levels of MRP6 mRNA.

This R1141X mutation results either in an (MRP6) ABCC6 protein lacking 362 amino acids at the C-terminal domain (Fig. 3) or a null allele, produced through nonsense

mediated decay of a truncated (MRP6) *ABCC6* mRNA. Indeed, an analysis of steady state (MRP6) *ABCC6* mRNA levels in skin fibroblasts from PXE patients of this Italian pedigree indicated the absence of detectable (MRP6) *ABCC6* mRNA, suggesting that the homozygous R1141X mutation results in the total loss of MRP6 gene product rather than the production of a truncated protein. R1141X was also found in a homozygous state in unrelated patients with autosomal recessive PXE from the United Kingdom and Belgium. Haplotype analysis of the PXE locus in families with the R1141X mutation revealed that this mutation is travelling within different haplotypes, suggesting that R1141X may be a recurrent mutation.

In two families with a recessive form of PXE from the United Kingdom and the United States, PXE patients were found to be compound heterozygotes. Affected individuals carried a substitution (TVS21+1G>T) affecting the donor- splice site of exon 21 of (MRP6) *ABCC6,* in association with either the nonsense RI141X substitution in exon 24 or a missense mutation, R1138Q also in exon 24. The splice site mutation occurred at the donor invariant dinucleotide and lowered the splice potential score from 72.1 to 53.8. Several other missense variants (Table 2) were also found within exon 24 and 28 of the (MRP6) *ABCC6* gene. These single nucleotide substitutions, none of which were detected in the control panel of 200 alleles, occurred within highly conserved coding domains, particularly the domain in exon 28 encoding the Walker A region of the second ATP binding fold (Fig. 2).

All the detected homozygous or compound heterozygous mutations were found to be associated with autosomal recessive PXE. One missense mutation (3961G>A) was observed in a family with an apparently dominant form of PXE. All the other heterozygous alterations were detected in individuals with sporadic PXE. The mode of inheritance of these sporadic PXE cases is presently unknown.

Elastic fibers within elastic tissues such as skin and the arterial wall are fragmented and calcified in PXE patients. Dermal and vascular elastic fiber calcification is patchy and does not involve all elastic fibers in these tissues. Therefore, without wishing to be bound by any particular theory, calcification of elastic fibers in PXE is probably therefore, a secondary consequence of a primary defect of either elastic fiber assembly or the interaction of elastic fibers with other extracellular matrix components.

Accordingly, MRP6 function is more likely to be related to fiber assembly or matrix interactions than calcium transport. Another possibility is that the maintenance of the integrity of normal elastic fibers, extracellular matrix polymers subject to constant mechanical stress, is modulated by (MRP6) ABCC6 in a way that has yet to be explained.

Polymorphic markers in genes encoding known elastic fiber proteins (tropoelastin, lysyl oxidase, fibrillin 1 and 2) were used in a linkage and sib pair analysis, performed with families with both autosomal recessive (AR) and dominant (AD) forms of PXE. No obvious linkage between these markers and the PXE phenotype was found.

Table 2

nt change	Codon #	Effect	Location	Status
UNK gene polymorphisms				
33C>T	11	Ser to Ser	Exon 1	Hetero
***pM5* gene polymorphisms**				
2187C>T	729	Gly to Gly	Exon 19	Hetero
3241G>A	1081	Glu to Lys	Exon 28	Hetero
MRP-1 gene polymorphisms				
1062T>C	354	Asn to Asn	Exon 9	Hetero
1898G>A	633	Arg to Gln	Exon 14	Hetero
2001C>T	667	Ser to Ser	Exon 16	Hetero
2012G>T	671	Gly to Val	Exon 16	Both
4002G>A	1334	Ser to Ser	Exon 28	Hetero
IVS29-18delT	-	-	Intron 29	Hetero
(MRP6) **ABCC6 gene polymorphisms**				
549G>A	183	Leu to Leu	Exon 5	Hetero
IVS11-41A>G	-	-	Intron 11	Both

1841T>C	614	Val to Ala	Exon 14	Both
2490C>T	830	Ala to Ala	Exon19	Both
IVS25+90G>A	-	-	Intron25	Both
IVS27-46A>G	-	-	Intron 27	Hetero
IVS28+49C>T	-	-	Intron 28	Hetero
3'UTR+17G>A	-	-	3'UTR	Hetero
(MRP6) **ABCC6 gene mutations**				
IVS21+1G>T	-	mRNA splicing	Intron 21	Compound
3341G>C	1114	Arg to Pro	Exon 24	Homo
3413G>A	1138	Arg to Gln	Exon 24	Compound
3421C>T	1141	Arg to X	Exon 24	Compound+Both
3775delT	1259	Fram Shift	Exon 27	Hetero
3892G>T	1298	Val to Phe	Exon 28	Hetero
3904G>A	1302	Gly to Arg	Exon 28	Homo
3907G>C	1303	Ala to Pro	Exon 28	Hetero
3940C>T	1314	Arg to Trp	Exon 28	Homo
3961G>A	1321	Gly to Ile	Exon 28	Hetero

Table 2: A summary of all variants identified in the PXE locus in a cohort of 20 unrelated PXE patients. Nucleotide (nt) numbering was derived either from full length published cDNA sequences or from putative cDNA deduced from genomic DNA sequence. Hetero indicates that a variant was identified in a heterozygous state. Homo indicates that a variant was found in a homozygous state. Both, indicates that a variant was seen in both heterozygous and homozygous states. Compound, indicates that a variant was characterized as a compound heterozygote.

Example 3: Mutation detection in dominant pedigrees

The segregation of *ABCC6* mutations with the PXE phenotype was studied in three pedigrees with an apparent dominant inheritance. Two of the dominant families (families 1 and 3) presented three generations of individuals, while the remaining pedigree contained only two generations. In all 3 families, a heterozygous mutation, R1141X in exon 24, was found to segregate with the PXE phenotype. In the two-generation family (family 2), an apparent loss of heterozygosity of the R1141X allele was detected in the second generation of affected individuals (II-1 to -3). Several polymorphic variants in the surrounding exons and introns were subsequently analyzed by SSCP. Only one variant, V614A in exon 14, was found to be informative. These results suggested a heterozygous sub-microscopic deletion, which was paternally inherited. This deletion, with a breakpoint between exon 14 and 24, extended beyond exon 24, probably corresponds to a recurrent deletion recently characterized in 4 unrelated families. The latter deletion, confined to a region of the gene between intron 22 and 29 eliminated 16.5 kb of genomic DNA. Therefore, individuals II-1, II-2 and II-3 of family 2 have inherited compound heterozygote mutations, clearly indicating the recessive nature of PXE in this pedigree. Moreover, the phenotype displayed by the mother (Individual I-2) carrying a heterozygous allele R1141X, suggested the partial expression of the phenotype in an obligate carrier. Indeed, individual I-2 showed discreet skin lesions on the neck associated with a positive von Kossa staining of a skin biopsy-(from lesional skin) indicating the presence of calcium salt precipitates typical of PXE. No angioid streaks were reported for this obligate carrier and no cardiovascular examination has been performed yet. In the remaining families (family 1 and 3) no other mutations were found. However, the PXE phenotype of the family members dramatically varied with the generations, clearly suggesting either pseudo-dominance or partial penetrance in obligate carriers. In family 1, the paternal grandmother and the father presented discreet skin lesions on the neck region associated with a positive von Kossa staining of a skin biopsy (no other manifestation were diagnosed), while both children, although very young, had already visible signs of plaques of coalesced papules on the neck and angioids streaks following ocular examination. In family 3, the paternal grandfather was severely affected with lax and redundant skin, disciform scaring of the

retina (the vision is severely impaired at this stage) in addition to active gastrointestinal bleeding and intermittent claudication. The father was only diagnosed with a positive von Kossa staining of a skin biopsy while 3 of his children presented with the characteristic PXE skin lesions and angioid streaks.

Accordingly, heterozygote carriers of PXE mutations can develop PXE related phenotypes including sub-clinical manifestations of PXE. According to the invention, the penetrance of PXE lesions associated with a single mutant (MRP6) *ABCC6* allele is between about 10 to 20% of all carriers, based on the frequency of described AD PXE cases. Therefore, a pedigree with AR PXE presents sub-clinical manifestations of PXE in 10 to 20% of the obligate carriers. These carriers will be parents of an affected individual and 25% of the unaffected siblings.

Example 4: PXE Heterozygote Frequencies

Upon screening a small sample of the general population (150 normal individuals) as part of a control panel to verify whether nucleotide substitutions found in the (MRP6) *ABCC6* gene from PXE patients were indeed mutations, two heterozygote mutations were found in unrelated subjects. The first of these variants was a founder mutation (R1339C) only present in South African Afrikaners while the second substitution is a recurrent nonsense mutation (R1141X). R1141X is one of the four recurrent mutations that have been identified. These mutations are far more likely to be found in the general population than private mutations, which, in principle, can only be found in related individuals. The frequency of heterozygote carriers deduced from the presence of one recurrent mutation in the relatively small sample of the general population is 0.7%. However, four recurrent mutations have thus far been identified. Although each of the recurrent mutations is likely to have a different frequency, the frequency of carriers can be as high as 2.8%, which is consistent with the commonly accepted prevalence of heterozygote carriers in the general population (0.6 to 2.5%).

Based upon these frequency of heterozygotes and the predicted penetrance of the PXE phenotype in heterozygote carriers (10-20%), heterozygote carriers with PXE symptoms are expected at a frequency of about 0.25% of the general population. In a cohort of about 3000 individuals between 8 and 15 persons presenting cardiovascular,

ocular or dermal symptoms would be expected. These numbers provide a basis for a statistical analysis of the correlation between single (MRP6) *ABCC6* mutations and partial manifestations of PXE. Additional cohorts with clinically defined cardiovascular abnormalities such as the 1200 sib-pairs group from the Family Blood Pressure Program with hypertension, or the NHLBI Framingham study (http://www.nhlbi.nih.gov/about/framingham/) from which an appropriate cohort of 2400 to 4500 individuals is available, can be used to provide additional statistical significance.

Example 5: Creating a mouse knockout

To create a knock-out mouse for ABCC6 a neomycin resistance cassette is introduced between exons 28 and 29 as shown in Fig. 6. This results in the destruction of the second ATP binding domain whose Walker A domain is encoded by exon 28 and which is essential for the function of any ABC transporter.

Based on the cDNA sequence for the mouse ABCC6 gene (SEQ ID NO: 8), primers with restriction sites were designed to amplify genomic DNA and allow cloning into vector pPNT described in Tybulewicz et al., Cell vol. 65, 1153-1163, 1991. Specifically, a 2.2 kb DNA fragment from exon 26 to exon 28 is cloned into the unique BamHI of pPNT, and a 2.3 kb DNA fragment containing exon 29 to 30 was cloned in the XhoI site of pPNT. In the resulting construct, the neomycin cassette from the vector interrupts the reading frame of ABCC6 in exon 28 after the conserved Lysine in the walker A domain.

This construct will be linearized by NotI digestion and transfected into mouse 129 stem cells. The two resistance cassettes provided by the vector (TK and Neo) will allow screening for homologous recombination and knock out of an ABCC6 locus according to methods known in the art (see, for example, Tybulewicz et al., Cell vol. 65, 1153-1163, 1991).

The deletion construct shown in Fig. 6 will be transfected into *E. coli* and amounts of DNA sufficient for the targeted mutagenesis process will be produced. This construct will be inserted into embryonic cell lines and cells that incorporate the construct will be implanted into surrogate mothers and MRP6 null mice will be obtained according to methods known in the art.

According to the invention, the production of MRP6 null mice with symptoms resembling those of human PXE would provide further proof that mutations at the MRP6 locus are responsible for PXE. However, a more important use for MRP6 null mice, or mice that are carriers of an MRP6 deletion (heterozygotes having an allele with the MRP6 deletion and a wild-type MRP6 allele) is to provide an animal model to study the development and progression of PXE, and to provide an animal model useful in the development of therapeutic approaches (including identifying therapeutic drugs) to treat existing PXE or to prevent or reduce the symptoms of PXE before they develop.

Example 6: Examples of oligonucleotide probes and probes useful to detect PXE associated MRP mutations.

Various probes corresponding to regions of specific mutations in *ABCC6* are used in standard oligonucleotide hybridization, in oligonucleotide array or nucleic acid chip assays (see www.brownlab.stanford.edu), and in PCR-based techniques for the detection of PXE. Each of the mutations shown below are indicative of a mutation in the ABCC6 gene that leads to PXE.

In a preferred embodiment, the probe shown in SEQ ID NO: 10 is used for the detection of a G to A mutation in Exon 24 of the ABCC6 gene.

CAGTGGTCCAGGGCATTCCGA (SEQ ID NO: 10)

In another embodiment, the probe shown in SEQ ID NO: 11 is used for the detection of a C to T mutation in Exon 24 of the ABCC6 gene.

CAGTGGTCCGGGCATTCTGA (SEQ ID NO: 11)

In yet another embodiment of the invention, the probe in SEQ ID NO: 12 is used for the detection of a G to C mutation in Exon 24 of the ABCC6 gene.

GACCGTTGGAGTCAGCCAGCTACTCG (SEQ ID NO: 12).

In another embodiment of the invention, the probe in SEQ ID NO: 13 is used for the detection of a C to G mutation in Exon 24 of the ABCC6 gene.

GACCCTTGGAGTCAGCCAGCTACTGG (SEQ ID NO: 13)

In another embodiment, the following probes are used for the detection of specific mutations in Exon 26 of the ABCC6 gene.

In a preferred embodiment of the invention, the probe in SEQ ID NO: 14 is used for the detection of a C to T mutation in Exon 26 of the ABCC6 gene.

GGATGTAGGACTATGCCTGGACGCCC (SEQ ID NO: 14)

In a preferred embodiment of the invention, the probe in SEQ ID NO: 15 is used for the detection of a G to C mutation in Exon 26 of the ABCC6 gene.

GGATGCAGGACTATGCCTGCACGCCC (SEQ ID NO: 15)

In yet another preferred embodiment of the invention, specific mutations in Exon 27 of the ABCC6 gene are detected using the probes shown below.

In a preferred embodiment of the invention, the probe in SEQ ID NO: 16 is used for the detection of a C to A substitution in Exon 27 of the ABCC6 gene

TGCAGCTAAGCCCCCCTGGC (SEQ ID NO: 16)

The probe sequence in SEQ ID NO: 17 is used for the detection of a deletion in Exon 27 of the ABCC6 gene.

TGCAGCTCAGCCCCCCGGC (SEQ ID NO: 17)

In yet another embodiment of the invention, the probe in SEQ ID NO: 18 is used for the detection of a G to A mutation in Exon 27 of the ABCC6 gene.

GCTCCAAGCTCCCTGGAGGC (SEQ ID NO: 18)

Mutations in Exon 28 of the ABCC6 gene in patients are detected using the probes shown in SEQ ID NOs. 19, 20, 21, 22, 23, 24 and 25.

In a preferred embodiment of the invention, the probe in SEQ ID. 19 is used for the detection of a C to T mutation in Exon 28 of the ABCC6 gene.

CTGTGGCTCCAGGAGGCAGCTGAGGGTGGG (SEQ ID NO: 19)

In yet another preferred embodiment of the invention, the probe in SEQ ID NO: 20 is used for the detection of a G to A mutation in Exon 28 of the ABCC6 gene.

CTGCAGCTCCAGGAGGCAGCTGAGGGTGGG (SEQ ID NO: 20)

Similarly, in a preferred embodiment of the invention, the probe in SEQ ID NO: 21 is used for the detection of a G to A mutation in a different region of Exon 28 of the ABCC6 gene.

CTGCGGCTCCAGGAGGCAGCTGAGAGTGGG (SEQ ID NO: 21)

Probes in SEQ ID NOs. 22, 23, 24 and 25 are used for the detection of additional specific mutations in Exon 28 of the ABCC6 gene.

GTGGGCATCTTTGGCAGGACCGGGG (SEQ ID NO: 22)

GTGGGCATCGTTGGCAGGACTGGGG (SEQ ID NO: 23)

GTGGGCATCTTTGGCAGGACCAGGG (SEQ ID NO: 24)

GTGGGCATCTTTGGCAGGACCGGGC (SEQ ID NO: 25)

EQUIVALENTS

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

INCORPORATION BY REFERENCE

Each of the patent documents and scientific publications disclosed herein is incorporated by reference into this application in its entirety.

What is claimed is:

CLAIMS

1. A method for screening for the presence of a PXE mutation, the method comprising the steps of:

interrogating an MRP6 nucleic acid in a patient sample for the presence of a PXE mutation; and,

identifying a positive screen as the presence of said mutation.

2. The method according to claim 1, wherein the said patient sample is selected from the group consisting of blood, saliva, amniotic fluid, and tissue.

3. The method according to claim 2, wherein the said patient sample is blood.

4. The method according to claim 1, wherein said interrogating step is a nucleic acid sequence scanning assay.

5. The method according to claim 4, wherein said scanning assay is selected from the group consisting of SSCP, DGGE, RFLP, LCR, DHPLC, and enzymatic cleavage.

6. The method according to claim 1, wherein said interrogating step is a specific mutation detection assay.

7. The method according to claim 6, wherein said detection assay is selected from the group consisting of oligonucleotide hybridization and primer extension assays.

8. The method according to claim 1, wherein said interrogating step is a nucleic acid sequencing assay.

9. The method according to claim 1, wherein said assay detects the presence of a mutation selected from the group consisting of a deletion, a substitution, an insertion, and a rearrangement.

10. The method according to claim 1, wherein said mutation is a mutation in codon 1141.

11. The method according to claim 1, wherein said mutation is a deletion of base 3775.

12. The method according to claim 1, wherein said mutation is a non-conserved amino acid substitution.

13. The method according to claim 1, wherein said mutation is in a codon selected from the group consisting of 1114, 1138, 1141, 1298, 1302, 1303, 1314, and 1321.

14. The method according to claim 1, wherein said mutation is in a splice site in an intron.

15. The method according to claim 1, wherein said mutation is in the promoter region of the MRP6 gene.

16. The method according to claim 1, wherein said mutation is in a polyA site of the MRP6 gene

17. The method according to claim 1, wherein said nucleic acid is selected from the group consisting of mRNA, genomic DNA, and cDNA.

18. A method for screening for the presence of a PXE mutation, the method comprising the steps of:

detecting MRP6 expression; and,

identifying a positive screen as one that detects a level of MRP6 expression that is lower than normal MRP6 expression.

19. The method of claim 18, wherein said detecting step detects MRP6 mRNA.

20. The method of claim 18, wherein said detecting step detects MRP6 protein.

21. The method according to claim 20, wherein said detecting step is an antibody-based assay.

22. The method according to claim 21, wherein said assay is selected from the group consisting of an ELISA and a Western blot.

23. A method for screening for the presence of a PXE mutation, the method comprising the steps of:

interrogating an MRP6 protein in a patient for the presence of a PXE protein change; and,

identifying a positive screen as the presence of said change.

24. The method according to claim 23, wherein said interrogating step measures the size of the MRP6 protein.

25. The method according to claim 1, wherein said assay detects the presence of a mutation in an exon of the MRP6 gene.

26. The method according to claim 25, wherein said exon is selected from exons 1-31 of the MRP6 gene

27. The method according to claim 1, wherein said interrogating step is a hybridization assay.

28. An oligonucleotide for use in an assay to detect a PXE associated mutation, wherein said oligonucleotide hybridizes to a PXE mutation in an MRP6 nucleic acid under stringent conditions.

29. An oligonucleotide of claim 32, wherein said oligonucleotide is selected from the group consisting of the oligonucleotides of SEQ ID NO: 10 through SEQ ID NO: 25.

30. A method for identifying a patient at risk of having children with PXE, the method comprising the step of interrogating a patient sample for the presence of a PXE mutant MRP6 allele.

31. A method for identifying a patient at risk of developing a PXE associated symptom, the method comprising the step of interrogating a patient sample for the presence of a PXE mutant MRP6 allele.

32. A method for diagnosing PXE in a patient, the method comprising the step of interrogating a patient sample for the presence of two PXE mutant MRP6 alleles.

33. The method of claim 32, wherein said patient is a homozygous PXE patient.

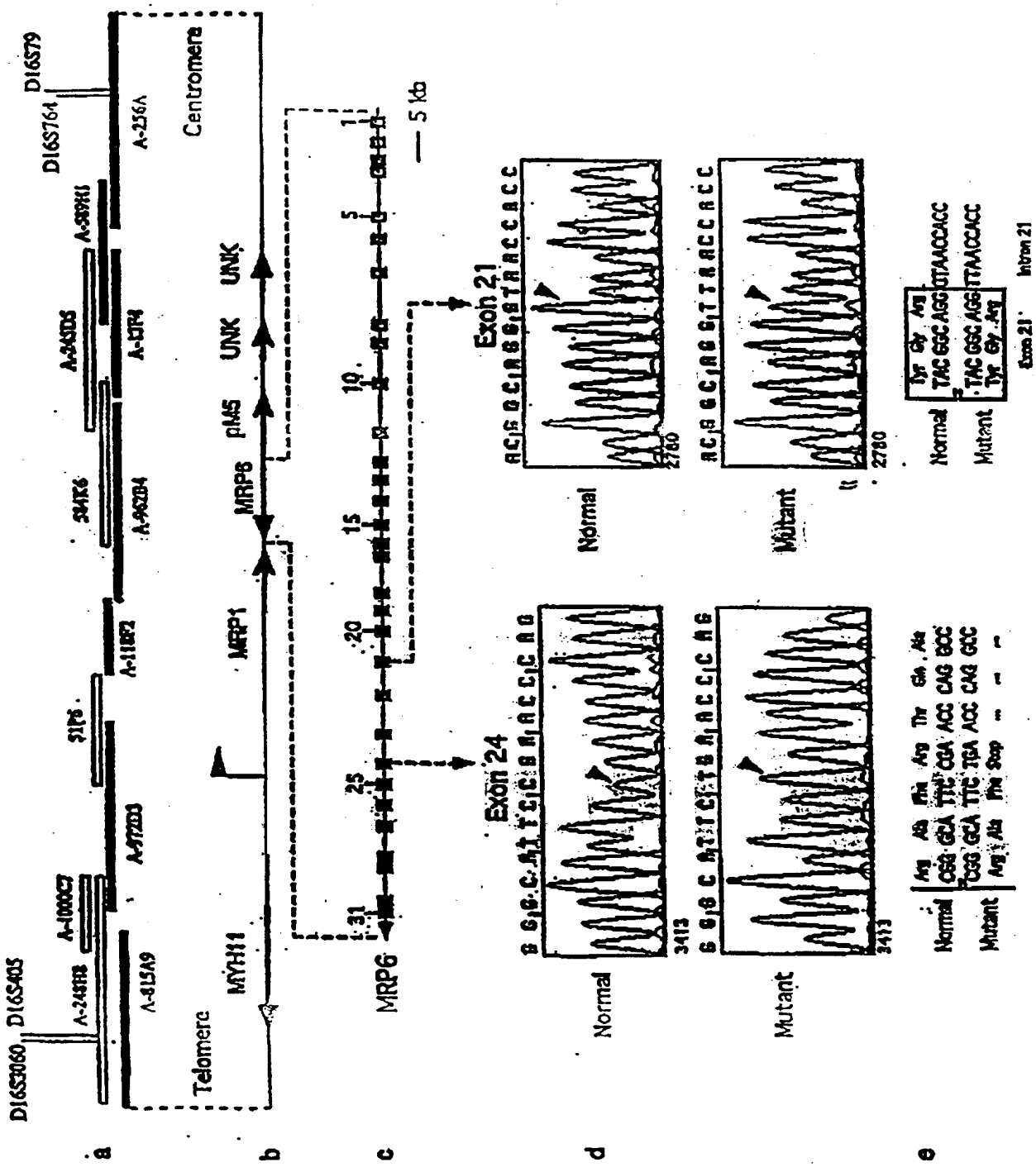
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Figure 1

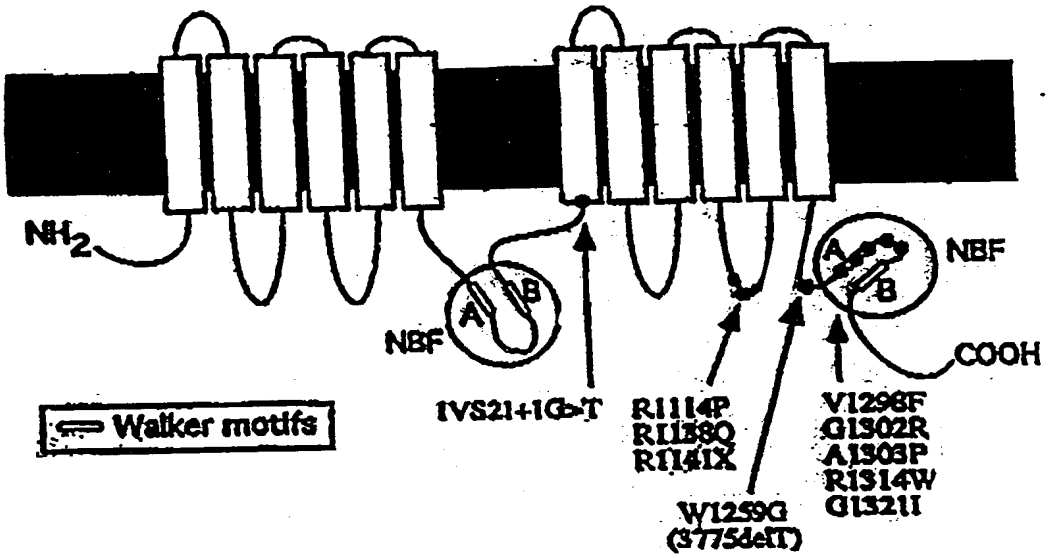
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Figure 2

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Figure 3

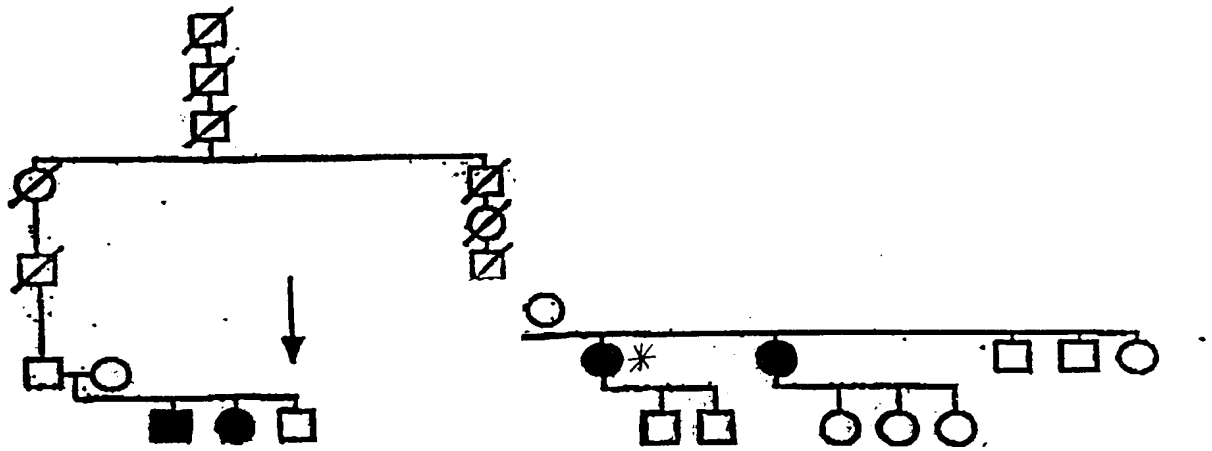

Figure 4

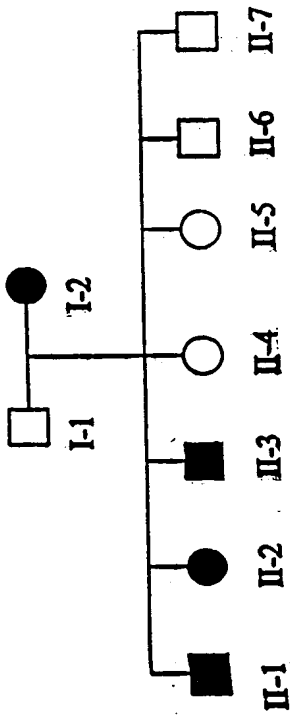

Figure 5

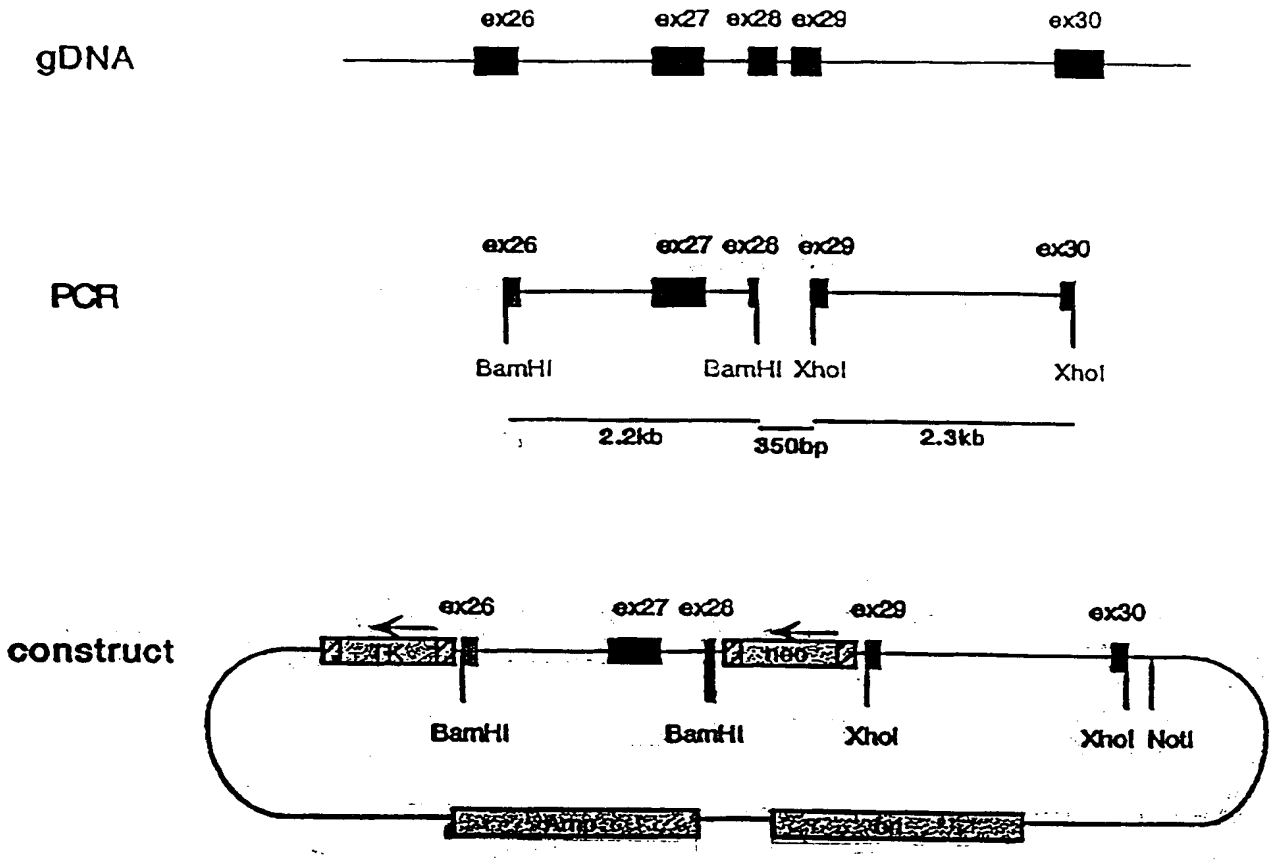
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Figure 6

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University of Hawaii

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Met Ala Ala Pro Ala Glu Pro Cys Ala Gly Gln Gly Val Trp Asn Gln
1               5                  10                  15

aca gag cct gaa cct gcc gcc acc agc ctg ctg agc ctg tgc ttc ctg       96
Thr Glu Pro Glu Pro Ala Ala Thr Ser Leu Leu Ser Leu Cys Phe Leu
            20                  25                  30

aga aca gca ggg gtc tgg gta ccc ccc atg tac ctc tgg gtc ctt ggt      144
Arg Thr Ala Gly Val Trp Val Pro Pro Met Tyr Leu Trp Val Leu Gly
        35                  40                  45

ccc atc tac ctc ctc ttc atc cac cac cat ggc cgg ggc tac ctg tgg      192
Pro Ile Tyr Leu Leu Phe Ile His His His Gly Arg Gly Tyr Leu Trp
    50                  55                  60
```

```
atg tcc cca ctc ttc aaa gcc aag atg gtg ctt gga ttc gcc ctc ata      240
Met Ser Pro Leu Phe Lys Ala Lys Met Val Leu Gly Phe Ala Leu Ile
65                  70                  75                  80

gtc ctg tgt acc tcc agc gtg gct gtc gct ctt tgg aaa atc caa cag      288
Val Leu Cys Thr Ser Ser Val Ala Val Ala Leu Trp Lys Ile Gln Gln
                85                  90                  95

gga acg cct gag gcc cca gaa ttc ctc att cat cct act gtg tgg ctc      336
Gly Thr Pro Glu Ala Pro Glu Phe Leu Ile His Pro Thr Val Trp Leu
            100                 105                 110

acc acg atg agc ttc gca gtg ttc ctg att cac acc gag agg aaa aag      384
Thr Thr Met Ser Phe Ala Val Phe Leu Ile His Thr Glu Arg Lys Lys
        115                 120                 125

gga gtc cag tca tct gga gtg ctg ttt ggt tac tgg ctt ctc tgc ttt      432
Gly Val Gln Ser Ser Gly Val Leu Phe Gly Tyr Trp Leu Leu Cys Phe
    130                 135                 140

gtc ttg cca gct acc aac gct gcc cag cag gcc tcc gga gcg ggc ttc      480
Val Leu Pro Ala Thr Asn Ala Ala Gln Gln Ala Ser Gly Ala Gly Phe
145                 150                 155                 160

cag agc gac cct gtc cgc cac ctg tcc acc tac cta tgc ctg tct ctg      528
Gln Ser Asp Pro Val Arg His Leu Ser Thr Tyr Leu Cys Leu Ser Leu
                165                 170                 175

gtg gtg gca cag ttt gtg ctg tcc tgc ctg gcg gat caa ccc ccc ttc      576
Val Val Ala Gln Phe Val Leu Ser Cys Leu Ala Asp Gln Pro Pro Phe
            180                 185                 190

ttc cct gaa gac ccc cag cag tct aac ccc tgt cca gag act ggg gca      624
Phe Pro Glu Asp Pro Gln Gln Ser Asn Pro Cys Pro Glu Thr Gly Ala
        195                 200                 205

gcc ttc ccc tcc aaa gcc acg ttc tgg tgg gtt tct ggc ctg gtc tgg      672
Ala Phe Pro Ser Lys Ala Thr Phe Trp Trp Val Ser Gly Leu Val Trp
    210                 215                 220

agg gga tac agg agg cca ctg aga cca aaa gac ctc tgg tcg ctt ggg      720
Arg Gly Tyr Arg Arg Pro Leu Arg Pro Lys Asp Leu Trp Ser Leu Gly
225                 230                 235                 240

aga gaa aac tcc tca gaa gaa ctt gtt tcc cgg ctt gaa aag gag tgg      768
Arg Glu Asn Ser Ser Glu Glu Leu Val Ser Arg Leu Glu Lys Glu Trp
                245                 250                 255

atg agg aac cgc agt gca gcc cgg agg cac aac aag gca ata gca ttt      816
Met Arg Asn Arg Ser Ala Ala Arg Arg His Asn Lys Ala Ile Ala Phe
            260                 265                 270

aaa agg aaa ggc ggc agt ggc atg aag gct cca gag acc gag ccc ttc      864
Lys Arg Lys Gly Gly Ser Gly Met Lys Ala Pro Glu Thr Glu Pro Phe
        275                 280                 285

cta cgg caa gaa ggg agc cag tgg cgc cca ctg ctg aag gcc atc tgg      912
```

```
Leu Arg Gln Glu Gly Ser Gln Trp Arg Pro Leu Leu Lys Ala Ile Trp
    290                 295                 300

cag gtg ttc cat tct acc ttc ctc ctg ggg acc ctc agc ctc atc atc      960
Gln Val Phe His Ser Thr Phe Leu Leu Gly Thr Leu Ser Leu Ile Ile
305                 310                 315                 320

agt gat gtc ttc agg ttc act gtc ccc aag ctg ctc agc ctt ttc ctg     1008
Ser Asp Val Phe Arg Phe Thr Val Pro Lys Leu Leu Ser Leu Phe Leu
                325                 330                 335

gag ttt att ggt gat ccc aag cct cca gcc tgg aag ggc tac ctc ctc     1056
Glu Phe Ile Gly Asp Pro Lys Pro Pro Ala Trp Lys Gly Tyr Leu Leu
            340                 345                 350

gcc gtg ctg atg ttc ctc tca gcc tgc ctg caa acg ctg ttt gag cag     1104
Ala Val Leu Met Phe Leu Ser Ala Cys Leu Gln Thr Leu Phe Glu Gln
        355                 360                 365

cag aac atg tac agg ctc aag gtg ctg cag atg agg ttg cgg tcg gcc     1152
Gln Asn Met Tyr Arg Leu Lys Val Leu Gln Met Arg Leu Arg Ser Ala
    370                 375                 380

atc act ggc ctg gtg tac aga aag gtc ctg gct ctg tcc agc ggc tcc     1200
Ile Thr Gly Leu Val Tyr Arg Lys Val Leu Ala Leu Ser Ser Gly Ser
385                 390                 395                 400

aga aag gcc agt gcg gtg ggt gat gtg gtc aat ctg gtg tcc gtg gac     1248
Arg Lys Ala Ser Ala Val Gly Asp Val Val Asn Leu Val Ser Val Asp
                405                 410                 415

gtg cag cgg ctg acc gag agc gtc ctc tac ctc aac ggg ctg tgg ctg     1296
Val Gln Arg Leu Thr Glu Ser Val Leu Tyr Leu Asn Gly Leu Trp Leu
            420                 425                 430

cct ctc gtc tgg atc gtg gtc tgc ttc gtc tat ctc tgg cag ctc ctg     1344
Pro Leu Val Trp Ile Val Val Cys Phe Val Tyr Leu Trp Gln Leu Leu
        435                 440                 445

ggg ccc tcc gcc ctc act gcc atc gct gtc ttc ctg agc ctc ctc cct     1392
Gly Pro Ser Ala Leu Thr Ala Ile Ala Val Phe Leu Ser Leu Leu Pro
    450                 455                 460

ctg aat ttc ttc atc tcc aag aaa agg aac cac cat cag gag gag caa     1440
Leu Asn Phe Phe Ile Ser Lys Lys Arg Asn His His Gln Glu Glu Gln
465                 470                 475                 480

atg agg cag aag gac tca cgg gca cgg ctc acc agc tct atc ctc agg     1488
Met Arg Gln Lys Asp Ser Arg Ala Arg Leu Thr Ser Ser Ile Leu Arg
                485                 490                 495

aac tcg aag acc atc aag ttc cat ggc tgg gag gga gcc ttt ctg gac     1536
Asn Ser Lys Thr Ile Lys Phe His Gly Trp Glu Gly Ala Phe Leu Asp
            500                 505                 510

aga gtc ctg ggc atc cga ggc cag gag ctg ggc gcc ttg cgg acc tcc     1584
Arg Val Leu Gly Ile Arg Gly Gln Glu Leu Gly Ala Leu Arg Thr Ser
```

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        515                 520                 525

ggc ctc ctc ttc tct gtg tcg ctg gtg tcc ttc caa gtg tct aca ttt     1632
Gly Leu Leu Phe Ser Val Ser Leu Val Ser Phe Gln Val Ser Thr Phe
    530                 535                 540

ctg gtc gca ctg gtg gtg ttt gct gtc cac act ctg gtg gcc gag aat     1680
Leu Val Ala Leu Val Val Phe Ala Val His Thr Leu Val Ala Glu Asn
545                 550                 555                 560

gct atg aat gca gag aaa gcc ttt gtg act ctc aca gtt ctc aac atc     1728
Ala Met Asn Ala Glu Lys Ala Phe Val Thr Leu Thr Val Leu Asn Ile
                565                 570                 575

ctc aac aag gcc cag gct ttc ctg ccc ttc tcc atc cac tcc ctc gtc     1776
Leu Asn Lys Ala Gln Ala Phe Leu Pro Phe Ser Ile His Ser Leu Val
            580                 585                 590

cag gcc cgg gtg tcc ttt gac cgt ctg gtc acc ttc ctc tgc ctg gaa     1824
Gln Ala Arg Val Ser Phe Asp Arg Leu Val Thr Phe Leu Cys Leu Glu
        595                 600                 605

gaa gtt gac cct ggt gtc gta gac tca agt tcc tct gga agc gct gcc     1872
Glu Val Asp Pro Gly Val Val Asp Ser Ser Ser Ser Gly Ser Ala Ala
    610                 615                 620

ggg aag gat tgc atc acc ata cac agt gcc acc ttc gcc tgg tcc cag     1920
Gly Lys Asp Cys Ile Thr Ile His Ser Ala Thr Phe Ala Trp Ser Gln
625                 630                 635                 640

gaa agc cct ccc tgc ctc cac aga ata aac ctc acg gtg ccc cag ggc     1968
Glu Ser Pro Pro Cys Leu His Arg Ile Asn Leu Thr Val Pro Gln Gly
                645                 650                 655

tgt ctg ctg gct gtt gtc ggt cca gtg ggg gca ggg aag tcc tcc ctg     2016
Cys Leu Leu Ala Val Val Gly Pro Val Gly Ala Gly Lys Ser Ser Leu
            660                 665                 670

ctg tcc gcc ctc ctt ggg gag ctg tca aag gtg gag ggg ttc gtg agc     2064
Leu Ser Ala Leu Leu Gly Glu Leu Ser Lys Val Glu Gly Phe Val Ser
        675                 680                 685

atc gag ggt gct gtg gcc tac gtg ccc cag gag gcc tgg gtg cag aac     2112
Ile Glu Gly Ala Val Ala Tyr Val Pro Gln Glu Ala Trp Val Gln Asn
    690                 695                 700

acc tct gtg gta gag aat gtg tgc ttc ggg cag gag ctg gac cca ccc     2160
Thr Ser Val Val Glu Asn Val Cys Phe Gly Gln Glu Leu Asp Pro Pro
705                 710                 715                 720

tgg ctg gag aga gta cta gaa gcc tgt gcc ctg cag cca gat gtg gac     2208
Trp Leu Glu Arg Val Leu Glu Ala Cys Ala Leu Gln Pro Asp Val Asp
                725                 730                 735

agc ttc cct gag gga atc cac act tca att ggg gag cag ggc atg aat     2256
Ser Phe Pro Glu Gly Ile His Thr Ser Ile Gly Glu Gln Gly Met Asn
            740                 745                 750
```

```
ctc tcc gga ggc cag aag cag cgg ctg agc ctg gcc cgg gct gta tac      2304
Leu Ser Gly Gly Gln Lys Gln Arg Leu Ser Leu Ala Arg Ala Val Tyr
        755                 760                 765

aga aag gca gct gtg tac ctg ctg gat gac ccc ctg gcg gcc ctg gat      2352
Arg Lys Ala Ala Val Tyr Leu Leu Asp Asp Pro Leu Ala Ala Leu Asp
    770                 775                 780

gcc cac gtt ggc cag cat gtc ttc aac cag gtc att ggg cct ggt ggg      2400
Ala His Val Gly Gln His Val Phe Asn Gln Val Ile Gly Pro Gly Gly
785                 790                 795                 800

cta ctc cag gga aca aca cgg att ctc gtg acg cac gca ctc cac atc      2448
Leu Leu Gln Gly Thr Thr Arg Ile Leu Val Thr His Ala Leu His Ile
                805                 810                 815

ctg ccc cag gct gat tgg atc ata gtg ctg gca aat ggg gcc atc gca      2496
Leu Pro Gln Ala Asp Trp Ile Ile Val Leu Ala Asn Gly Ala Ile Ala
            820                 825                 830

gag atg ggt tcc tac cag gag ctt ctg cag agg aag ggg gcc ctc gtg      2544
Glu Met Gly Ser Tyr Gln Glu Leu Leu Gln Arg Lys Gly Ala Leu Val
        835                 840                 845

tgt ctt ctg gat caa gcc aga cag cca gga gat aga gga gaa gga gaa      2592
Cys Leu Leu Asp Gln Ala Arg Gln Pro Gly Asp Arg Gly Glu Gly Glu
    850                 855                 860

aca gaa cct ggg acc agc acc aag gac ccc aga ggc acc tct gca ggc      2640
Thr Glu Pro Gly Thr Ser Thr Lys Asp Pro Arg Gly Thr Ser Ala Gly
865                 870                 875                 880

agg agg ccc gag ctt aga cgc gag agg tcc atc aag tca gtc cct gag      2688
Arg Arg Pro Glu Leu Arg Arg Glu Arg Ser Ile Lys Ser Val Pro Glu
                885                 890                 895

aag gac cgt acc act tca gaa gcc cag aca gag gtt cct ctg gat gac      2736
Lys Asp Arg Thr Thr Ser Glu Ala Gln Thr Glu Val Pro Leu Asp Asp
            900                 905                 910

cct gac agg gca gga tgg cca gca gga aag gac agc atc caa tac ggc      2784
Pro Asp Arg Ala Gly Trp Pro Ala Gly Lys Asp Ser Ile Gln Tyr Gly
        915                 920                 925

agg gtg aag gcc aca gtg cac ctg gcc tac ctg cgt gcc gtg ggc acc      2832
Arg Val Lys Ala Thr Val His Leu Ala Tyr Leu Arg Ala Val Gly Thr
    930                 935                 940

ccc ctc tgc ctc tac gca ctc ttc ctc ttc ctc tgc cag caa gtg gcc      2880
Pro Leu Cys Leu Tyr Ala Leu Phe Leu Phe Leu Cys Gln Gln Val Ala
945                 950                 955                 960

tcc ttc tgc cgg ggc tac tgg ctg agc ctg tgg gcg gac gac cct gca      2928
Ser Phe Cys Arg Gly Tyr Trp Leu Ser Leu Trp Ala Asp Asp Pro Ala
                965                 970                 975
```

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gta ggt ggg cag cag acg cag gca gcc ctg cgt ggc ggg atc ttc ggg     2976
Val Gly Gly Gln Gln Thr Gln Ala Ala Leu Arg Gly Gly Ile Phe Gly
            980                 985                 990

ctc ctc ggc tgt ctc caa gcc att  ggg ctg ttt gcc tcc  atg gct gcg   3024
Leu Leu Gly Cys Leu Gln Ala Ile  Gly Leu Phe Ala Ser  Met Ala Ala
        995                 1000                1005

gtg ctc  cta ggt ggg gcc cgg  gca tcc agg ttg ctc  ttc cag agg      3069
Val Leu  Leu Gly Gly Ala Arg  Ala Ser Arg Leu Leu  Phe Gln Arg
    1010                1015                1020

ctc ctg  tgg gat gtg gtg cga  tct ccc atc agc ttc  ttt gag cgg      3114
Leu Leu  Trp Asp Val Val Arg  Ser Pro Ile Ser Phe  Phe Glu Arg
    1025                1030                1035

aca ccc  att ggt cac ctg cta  aac cgc ttc tcc aag  gag aca gac      3159
Thr Pro  Ile Gly His Leu Leu  Asn Arg Phe Ser Lys  Glu Thr Asp
    1040                1045                1050

acg gtt  gac gtg gac att cca  gac aaa ctc cgg tcc  ctg ctg atg      3204
Thr Val  Asp Val Asp Ile Pro  Asp Lys Leu Arg Ser  Leu Leu Met
    1055                1060                1065

tac gcc  ttt gga ctc ctg gag  gtc agc ctg gtg gtg  gca gtg gct      3249
Tyr Ala  Phe Gly Leu Leu Glu  Val Ser Leu Val Val  Ala Val Ala
    1070                1075                1080

acc cca  ctg gcc act gtg gcc  atc ctg cca ctg ttt  ctc ctc tac      3294
Thr Pro  Leu Ala Thr Val Ala  Ile Leu Pro Leu Phe  Leu Leu Tyr
    1085                1090                1095

gct ggg  ttt cag agc ctg tat  gtg gtt agc tca tgc  cag ctg aga      3339
Ala Gly  Phe Gln Ser Leu Tyr  Val Val Ser Ser Cys  Gln Leu Arg
    1100                1105                1110

cgc ttg  gag tca gcc agc tac  tcg tct gtc tgc tcc  cac atg gct      3384
Arg Leu  Glu Ser Ala Ser Tyr  Ser Ser Val Cys Ser  His Met Ala
    1115                1120                1125

gag acg  ttc cag ggc agc aca  gtg gtc cgg gca ttc  cga acc cag      3429
Glu Thr  Phe Gln Gly Ser Thr  Val Val Arg Ala Phe  Arg Thr Gln
    1130                1135                1140

gcc ccc  ttt gtg gct cag aac  aat gct cgc gta gat  gaa agc cag      3474
Ala Pro  Phe Val Ala Gln Asn  Asn Ala Arg Val Asp  Glu Ser Gln
    1145                1150                1155

agg atc  agt ttc ccg cga ctg  gtg gct gac agg tgg  ctt gcg gcc      3519
Arg Ile  Ser Phe Pro Arg Leu  Val Ala Asp Arg Trp  Leu Ala Ala
    1160                1165                1170

aat gtg  gag ctc ctg ggg aat  ggc ctg gtg ttt gca  gct gcc acg      3564
Asn Val  Glu Leu Leu Gly Asn  Gly Leu Val Phe Ala  Ala Ala Thr
    1175                1180                1185

tgt gct  gtg ctg agc aaa gcc  cac ctc agt gct ggc  ctc gtg ggc      3609
```

```
Cys Ala  Val Leu Ser Lys Ala  His Leu Ser Ala Gly  Leu Val Gly
    1190                 1195                 1200

ttc tct  gtc tct gct gcc ctc  cag gtg acc cag aca  ctg cag tgg      3654
Phe Ser  Val Ser Ala Ala Leu  Gln Val Thr Gln Thr  Leu Gln Trp
    1205                 1210                 1215

gtt gtt  cgc aac tgg aca gac  cta gag aac agc atc  gtg tca gtg      3699
Val Val  Arg Asn Trp Thr Asp  Leu Glu Asn Ser Ile  Val Ser Val
    1220                 1225                 1230

gag cgg  atg cag gac tat gcc  tgg acg ccc aag gag  gct ccc tgg      3744
Glu Arg  Met Gln Asp Tyr Ala  Trp Thr Pro Lys Glu  Ala Pro Trp
    1235                 1240                 1245

agg ctg  ccc aca tgt gca gct  cag ccc ccc tgg cct  cag ggc ggg      3789
Arg Leu  Pro Thr Cys Ala Ala  Gln Pro Pro Trp Pro  Gln Gly Gly
    1250                 1255                 1260

cag atc  gag ttc cgg gac ttt  ggg cta aga tac cga  cct gag ctc      3834
Gln Ile  Glu Phe Arg Asp Phe  Gly Leu Arg Tyr Arg  Pro Glu Leu
    1265                 1270                 1275

ccg ctg  gct gtg cag ggc gtg  tcc ttc aag atc cac  gca gga gag      3879
Pro Leu  Ala Val Gln Gly Val  Ser Phe Lys Ile His  Ala Gly Glu
    1280                 1285                 1290

aag gtg  ggc atc gtt ggc agg  acc ggg gca ggg aag  tcc tcc ctg      3924
Lys Val  Gly Ile Val Gly Arg  Thr Gly Ala Gly Lys  Ser Ser Leu
    1295                 1300                 1305

gcc agt  ggg ctg ctg cgg ctc  cag gag gca gct gag  ggt ggg atc      3969
Ala Ser  Gly Leu Leu Arg Leu  Gln Glu Ala Ala Glu  Gly Gly Ile
    1310                 1315                 1320

tgg atc  gac ggg gtc ccc att  gcc cac gtg ggg ctg  cac aca ctg      4014
Trp Ile  Asp Gly Val Pro Ile  Ala His Val Gly Leu  His Thr Leu
    1325                 1330                 1335

cgc tcc  agg atc agc atc atc  ccc cag gac ccc atc  ctg ttc cct      4059
Arg Ser  Arg Ile Ser Ile Ile  Pro Gln Asp Pro Ile  Leu Phe Pro
    1340                 1345                 1350

ggc tct  ctg cgg atg aac ctc  gac ctg ctg cag gag  cac tcg gac      4104
Gly Ser  Leu Arg Met Asn Leu  Asp Leu Leu Gln Glu  His Ser Asp
    1355                 1360                 1365

gag gct  atc tgg gca gcc ctg  gag acg gtg cag ctc  aaa gcc ttg      4149
Glu Ala  Ile Trp Ala Ala Leu  Glu Thr Val Gln Leu  Lys Ala Leu
    1370                 1375                 1380

gtg gcc  agc ctg ccc ggc cag  ctg cag tac aag tgt  gct gac cga      4194
Val Ala  Ser Leu Pro Gly Gln  Leu Gln Tyr Lys Cys  Ala Asp Arg
    1385                 1390                 1395

ggc gag  gac ctg agc gtg ggc  cag aaa cag ctc ctg  tgt ctg gca      4239
Gly Glu  Asp Leu Ser Val Gly  Gln Lys Gln Leu Leu  Cys Leu Ala
```

```
    1400                1405                1410

cgt gcc  ctt ctc cgg aag acc  cag atc ctc atc ctg  gac gag gct      4284
Arg Ala  Leu Leu Arg Lys Thr  Gln Ile Leu Ile Leu  Asp Glu Ala
    1415                1420                1425

act gct  gcc gtg gac cct ggc  acg gag ctg cag atg  cag gcc atg      4329
Thr Ala  Ala Val Asp Pro Gly  Thr Glu Leu Gln Met  Gln Ala Met
    1430                1435                1440

ctc ggg  agc tgg ttt gca cag  tgc act gtg ctg ctc  att gcc cac      4374
Leu Gly  Ser Trp Phe Ala Gln  Cys Thr Val Leu Leu  Ile Ala His
    1445                1450                1455

cgc ctg  cgc tcc gtg atg gac  tgt gcc cgg gtt ctg  gtc atg gac      4419
Arg Leu  Arg Ser Val Met Asp  Cys Ala Arg Val Leu  Val Met Asp
    1460                1465                1470

aag ggg  cag gtg gca gag agc  ggc agc ccg gcc cag  ctg ctg gcc      4464
Lys Gly  Gln Val Ala Glu Ser  Gly Ser Pro Ala Gln  Leu Leu Ala
    1475                1480                1485

cag aag  ggc ctg ttt tac aga  ctg gcc cag gag tca  ggc ctg gtc      4509
Gln Lys  Gly Leu Phe Tyr Arg  Leu Ala Gln Glu Ser  Gly Leu Val
    1490                1495                1500

tga                                                               4512
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<210> 3
<211> 1503
<212> PRT
<213> Homo sapiens

<400> 3

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Met Ala Ala Pro Ala Glu Pro Cys Ala Gly Gln Gly Val Trp Asn Gln
1               5                   10                  15

Thr Glu Pro Glu Pro Ala Ala Thr Ser Leu Leu Ser Leu Cys Phe Leu
            20                  25                  30

Arg Thr Ala Gly Val Trp Val Pro Pro Met Tyr Leu Trp Val Leu Gly
        35                  40                  45

Pro Ile Tyr Leu Leu Phe Ile His His His Gly Arg Gly Tyr Leu Trp
    50                  55                  60

Met Ser Pro Leu Phe Lys Ala Lys Met Val Leu Gly Phe Ala Leu Ile
65                  70                  75                  80

Val Leu Cys Thr Ser Ser Val Ala Val Ala Leu Trp Lys Ile Gln Gln
```

85 90 95

Gly Thr Pro Glu Ala Pro Glu Phe Leu Ile His Pro Thr Val Trp Leu
100 105 110

Thr Thr Met Ser Phe Ala Val Phe Leu Ile His Thr Glu Arg Lys Lys
115 120 125

Gly Val Gln Ser Ser Gly Val Leu Phe Gly Tyr Trp Leu Leu Cys Phe
130 135 140

Val Leu Pro Ala Thr Asn Ala Ala Gln Gln Ala Ser Gly Ala Gly Phe
145 150 155 160

Gln Ser Asp Pro Val Arg His Leu Ser Thr Tyr Leu Cys Leu Ser Leu
165 170 175

Val Val Ala Gln Phe Val Leu Ser Cys Leu Ala Asp Gln Pro Pro Phe
180 185 190

Phe Pro Glu Asp Pro Gln Gln Ser Asn Pro Cys Pro Glu Thr Gly Ala
195 200 205

Ala Phe Pro Ser Lys Ala Thr Phe Trp Trp Val Ser Gly Leu Val Trp
210 215 220

Arg Gly Tyr Arg Arg Pro Leu Arg Pro Lys Asp Leu Trp Ser Leu Gly
225 230 235 240

Arg Glu Asn Ser Ser Glu Glu Leu Val Ser Arg Leu Glu Lys Glu Trp
245 250 255

Met Arg Asn Arg Ser Ala Ala Arg Arg His Asn Lys Ala Ile Ala Phe
260 265 270

Lys Arg Lys Gly Gly Ser Gly Met Lys Ala Pro Glu Thr Glu Pro Phe
275 280 285

Leu Arg Gln Glu Gly Ser Gln Trp Arg Pro Leu Leu Lys Ala Ile Trp
290 295 300

Gln Val Phe His Ser Thr Phe Leu Leu Gly Thr Leu Ser Leu Ile Ile
305 310 315 320

```
Ser Asp Val Phe Arg Phe Thr Val Pro Lys Leu Leu Ser Leu Phe Leu
                325                 330                 335

Glu Phe Ile Gly Asp Pro Lys Pro Pro Ala Trp Lys Gly Tyr Leu Leu
            340                 345                 350

Ala Val Leu Met Phe Leu Ser Ala Cys Leu Gln Thr Leu Phe Glu Gln
        355                 360                 365

Gln Asn Met Tyr Arg Leu Lys Val Leu Gln Met Arg Leu Arg Ser Ala
    370                 375                 380

Ile Thr Gly Leu Val Tyr Arg Lys Val Leu Ala Leu Ser Ser Gly Ser
385                 390                 395                 400

Arg Lys Ala Ser Ala Val Gly Asp Val Val Asn Leu Val Ser Val Asp
                405                 410                 415

Val Gln Arg Leu Thr Glu Ser Val Leu Tyr Leu Asn Gly Leu Trp Leu
            420                 425                 430

Pro Leu Val Trp Ile Val Val Cys Phe Val Tyr Leu Trp Gln Leu Leu
        435                 440                 445

Gly Pro Ser Ala Leu Thr Ala Ile Ala Val Phe Leu Ser Leu Leu Pro
    450                 455                 460

Leu Asn Phe Phe Ile Ser Lys Lys Arg Asn His His Gln Glu Glu Gln
465                 470                 475                 480

Met Arg Gln Lys Asp Ser Arg Ala Arg Leu Thr Ser Ser Ile Leu Arg
                485                 490                 495

Asn Ser Lys Thr Ile Lys Phe His Gly Trp Glu Gly Ala Phe Leu Asp
            500                 505                 510

Arg Val Leu Gly Ile Arg Gly Gln Glu Leu Gly Ala Leu Arg Thr Ser
        515                 520                 525

Gly Leu Leu Phe Ser Val Ser Leu Val Ser Phe Gln Val Ser Thr Phe
    530                 535                 540
```

```
Leu Val Ala Leu Val Val Phe Ala Val His Thr Leu Val Ala Glu Asn
545                 550                 555                 560

Ala Met Asn Ala Glu Lys Ala Phe Val Thr Leu Thr Val Leu Asn Ile
                565                 570                 575

Leu Asn Lys Ala Gln Ala Phe Leu Pro Phe Ser Ile His Ser Leu Val
            580                 585                 590

Gln Ala Arg Val Ser Phe Asp Arg Leu Val Thr Phe Leu Cys Leu Glu
        595                 600                 605

Glu Val Asp Pro Gly Val Val Asp Ser Ser Ser Ser Gly Ser Ala Ala
    610                 615                 620

Gly Lys Asp Cys Ile Thr Ile His Ser Ala Thr Phe Ala Trp Ser Gln
625                 630                 635                 640

Glu Ser Pro Pro Cys Leu His Arg Ile Asn Leu Thr Val Pro Gln Gly
                645                 650                 655

Cys Leu Leu Ala Val Val Gly Pro Val Gly Ala Gly Lys Ser Ser Leu
            660                 665                 670

Leu Ser Ala Leu Leu Gly Glu Leu Ser Lys Val Glu Gly Phe Val Ser
        675                 680                 685

Ile Glu Gly Ala Val Ala Tyr Val Pro Gln Glu Ala Trp Val Gln Asn
    690                 695                 700

Thr Ser Val Val Glu Asn Val Cys Phe Gly Gln Glu Leu Asp Pro Pro
705                 710                 715                 720

Trp Leu Glu Arg Val Leu Glu Ala Cys Ala Leu Gln Pro Asp Val Asp
                725                 730                 735

Ser Phe Pro Glu Gly Ile His Thr Ser Ile Gly Glu Gln Gly Met Asn
            740                 745                 750

Leu Ser Gly Gly Gln Lys Gln Arg Leu Ser Leu Ala Arg Ala Val Tyr
        755                 760                 765
```

```
Arg Lys Ala Ala Val Tyr Leu Leu Asp Asp Pro Leu Ala Ala Leu Asp
    770                 775                 780

Ala His Val Gly Gln His Val Phe Asn Gln Val Ile Gly Pro Gly Gly
785                 790                 795                 800

Leu Leu Gln Gly Thr Thr Arg Ile Leu Val Thr His Ala Leu His Ile
                805                 810                 815

Leu Pro Gln Ala Asp Trp Ile Ile Val Leu Ala Asn Gly Ala Ile Ala
            820                 825                 830

Glu Met Gly Ser Tyr Gln Glu Leu Leu Gln Arg Lys Gly Ala Leu Val
        835                 840                 845

Cys Leu Leu Asp Gln Ala Arg Gln Pro Gly Asp Arg Gly Glu Gly Glu
    850                 855                 860

Thr Glu Pro Gly Thr Ser Thr Lys Asp Pro Arg Gly Thr Ser Ala Gly
865                 870                 875                 880

Arg Arg Pro Glu Leu Arg Arg Glu Arg Ser Ile Lys Ser Val Pro Glu
                885                 890                 895

Lys Asp Arg Thr Thr Ser Glu Ala Gln Thr Glu Val Pro Leu Asp Asp
            900                 905                 910

Pro Asp Arg Ala Gly Trp Pro Ala Gly Lys Asp Ser Ile Gln Tyr Gly
        915                 920                 925

Arg Val Lys Ala Thr Val His Leu Ala Tyr Leu Arg Ala Val Gly Thr
    930                 935                 940

Pro Leu Cys Leu Tyr Ala Leu Phe Leu Phe Leu Cys Gln Gln Val Ala
945                 950                 955                 960

Ser Phe Cys Arg Gly Tyr Trp Leu Ser Leu Trp Ala Asp Asp Pro Ala
                965                 970                 975

Val Gly Gly Gln Gln Thr Gln Ala Ala Leu Arg Gly Gly Ile Phe Gly
            980                 985                 990

Leu Leu Gly Cys Leu Gln Ala Ile  Gly Leu Phe Ala Ser  Met Ala Ala
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995 1000 1005

Val Leu Leu Gly Gly Ala Arg Ala Ser Arg Leu Leu Phe Gln Arg
1010 1015 1020

Leu Leu Trp Asp Val Val Arg Ser Pro Ile Ser Phe Phe Glu Arg
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Thr Pro Ile Gly His Leu Leu Asn Arg Phe Ser Lys Glu Thr Asp
1040 1045 1050

Thr Val Asp Val Asp Ile Pro Asp Lys Leu Arg Ser Leu Leu Met
1055 1060 1065

Tyr Ala Phe Gly Leu Leu Glu Val Ser Leu Val Val Ala Val Ala
1070 1075 1080

Thr Pro Leu Ala Thr Val Ala Ile Leu Pro Leu Phe Leu Leu Tyr
1085 1090 1095

Ala Gly Phe Gln Ser Leu Tyr Val Val Ser Ser Cys Gln Leu Arg
1100 1105 1110

Arg Leu Glu Ser Ala Ser Tyr Ser Ser Val Cys Ser His Met Ala
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Glu Thr Phe Gln Gly Ser Thr Val Val Arg Ala Phe Arg Thr Gln
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Ala Pro Phe Val Ala Gln Asn Asn Ala Arg Val Asp Glu Ser Gln
1145 1150 1155

Arg Ile Ser Phe Pro Arg Leu Val Ala Asp Arg Trp Leu Ala Ala
1160 1165 1170

Asn Val Glu Leu Leu Gly Asn Gly Leu Val Phe Ala Ala Ala Thr
1175 1180 1185

Cys Ala Val Leu Ser Lys Ala His Leu Ser Ala Gly Leu Val Gly
1190 1195 1200

Phe Ser Val Ser Ala Ala Leu Gln Val Thr Gln Thr Leu Gln Trp
1205 1210 1215

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Val Val  Arg Asn Trp Thr Asp  Leu Glu Asn Ser Ile  Val Ser Val
    1220                 1225                 1230

Glu Arg  Met Gln Asp Tyr Ala  Trp Thr Pro Lys Glu  Ala Pro Trp
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Arg Leu  Pro Thr Cys Ala Ala  Gln Pro Pro Trp Pro  Gln Gly Gly
    1250                 1255                 1260

Gln Ile  Glu Phe Arg Asp Phe  Gly Leu Arg Tyr Arg  Pro Glu Leu
    1265                 1270                 1275

Pro Leu  Ala Val Gln Gly Val  Ser Phe Lys Ile His  Ala Gly Glu
    1280                 1285                 1290

Lys Val  Gly Ile Val Gly Arg  Thr Gly Ala Gly Lys  Ser Ser Leu
    1295                 1300                 1305

Ala Ser  Gly Leu Leu Arg Leu  Gln Glu Ala Ala Glu  Gly Gly Ile
    1310                 1315                 1320

Trp Ile  Asp Gly Val Pro Ile  Ala His Val Gly Leu  His Thr Leu
    1325                 1330                 1335

Arg Ser  Arg Ile Ser Ile Ile  Pro Gln Asp Pro Ile  Leu Phe Pro
    1340                 1345                 1350

Gly Ser  Leu Arg Met Asn Leu  Asp Leu Leu Gln Glu  His Ser Asp
    1355                 1360                 1365

Glu Ala  Ile Trp Ala Ala Leu  Glu Thr Val Gln Leu  Lys Ala Leu
    1370                 1375                 1380

Val Ala  Ser Leu Pro Gly Gln  Leu Gln Tyr Lys Cys  Ala Asp Arg
    1385                 1390                 1395

Gly Glu  Asp Leu Ser Val Gly  Gln Lys Gln Leu Leu  Cys Leu Ala
    1400                 1405                 1410

Arg Ala  Leu Leu Arg Lys Thr  Gln Ile Leu Ile Leu  Asp Glu Ala
    1415                 1420                 1425
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Thr Ala Ala Val Asp Pro Gly Thr Glu Leu Gln Met Gln Ala Met
1430 1435 1440

Leu Gly Ser Trp Phe Ala Gln Cys Thr Val Leu Leu Ile Ala His
1445 1450 1455

Arg Leu Arg Ser Val Met Asp Cys Ala Arg Val Leu Val Met Asp
1460 1465 1470

Lys Gly Gln Val Ala Glu Ser Gly Ser Pro Ala Gln Leu Leu Ala
1475 1480 1485

Gln Lys Gly Leu Phe Tyr Arg Leu Ala Gln Glu Ser Gly Leu Val
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Met Asn Ser Gly Arg Ser Met Ala Thr Pro Gly Glu Gln
1 5 10

tgc gcc ggc ctg agg gtc tgg aac cag aca gag cag gag cct gcg gcc 98
Cys Ala Gly Leu Arg Val Trp Asn Gln Thr Glu Gln Glu Pro Ala Ala
15 20 25

tat cac ttg ctc agc ctg tgc ttc gtg aga gcc gcc agc agc tgg gtg 146
Tyr His Leu Leu Ser Leu Cys Phe Val Arg Ala Ala Ser Ser Trp Val
30 35 40 45

ccc ccc atg tac ctc tgg gtc ctc ggc ccc atc tac ctt ctc tac atc 194
Pro Pro Met Tyr Leu Trp Val Leu Gly Pro Ile Tyr Leu Leu Tyr Ile
50 55 60

cat cgc cat ggc cgg tgc tac ctc cgg atg tcc cac ctc ttc aaa acc 242
His Arg His Gly Arg Cys Tyr Leu Arg Met Ser His Leu Phe Lys Thr
65 70 75

aaa atg gtg ctg ggc ttg gcc ctc atc ctt ctg tat acc ttc aac gtg 290
Lys Met Val Leu Gly Leu Ala Leu Ile Leu Leu Tyr Thr Phe Asn Val
80 85 90

gcc gtg cct ctg tgg agg atc cac cag ggc gtg ccc cag gcc cca gag 338
Ala Val Pro Leu Trp Arg Ile His Gln Gly Val Pro Gln Ala Pro Glu
95 100 105

ctt cta att cac cct act gtg tgg ctc acc acc atg agc ttt gcc acc 386
Leu Leu Ile His Pro Thr Val Trp Leu Thr Thr Met Ser Phe Ala Thr
110 115 120 125

ttt ctg atc cac atg gag aga agg aag gga gtc cgg tca tcc ggg gtg 434

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Phe Leu Ile His Met Glu Arg Arg Lys Gly Val Arg Ser Ser Gly Val
                130                 135                 140

ttg ttc ggg tac tgg ctg ctc tgc tgc atc ttg cca gga atc aac act      482
Leu Phe Gly Tyr Trp Leu Leu Cys Cys Ile Leu Pro Gly Ile Asn Thr
            145                 150                 155

gtg cag cag gcc tct gca ggg aac tta cgt cag gag ccc ctc cac cac      530
Val Gln Gln Ala Ser Ala Gly Asn Leu Arg Gln Glu Pro Leu His His
        160                 165                 170

ctg gcc acc tac ctg tgc ttg tcc ctg gtg gtg gct gag ctg gtg ctg      578
Leu Ala Thr Tyr Leu Cys Leu Ser Leu Val Val Ala Glu Leu Val Leu
    175                 180                 185

tcc tgt ctg gtg gac cag cca ccc ttc ttc tcg gaa gac tcc cag cca      626
Ser Cys Leu Val Asp Gln Pro Pro Phe Phe Ser Glu Asp Ser Gln Pro
190                 195                 200                 205

ttg aat ccg tgt cca gag gct gag gcc tcc ttt ccc tcc aag gcc atg      674
Leu Asn Pro Cys Pro Glu Ala Glu Ala Ser Phe Pro Ser Lys Ala Met
                210                 215                 220

ttc tgg tgg gcc tct gga ctg cta tgg agg ggc tac aaa aag ctg ctg      722
Phe Trp Trp Ala Ser Gly Leu Leu Trp Arg Gly Tyr Lys Lys Leu Leu
            225                 230                 235

gga cca aaa gac ctc tgg tca ctt ggg aga gaa aac tct tca gaa gaa      770
Gly Pro Lys Asp Leu Trp Ser Leu Gly Arg Glu Asn Ser Ser Glu Glu
        240                 245                 250

ctc gtt tcc cag ctg gaa aga gaa tgg agg aga agc tgc aat ggg ctg      818
Leu Val Ser Gln Leu Glu Arg Glu Trp Arg Arg Ser Cys Asn Gly Leu
    255                 260                 265

cca ggg cac aaa ggg cac agt agt gtg ggg gcc cct gag aca gag gcc      866
Pro Gly His Lys Gly His Ser Ser Val Gly Ala Pro Glu Thr Glu Ala
270                 275                 280                 285

ttc ctg cag cca gag agg agt cag agg ggc cca cta ctc agg gct atc      914
Phe Leu Gln Pro Glu Arg Ser Gln Arg Gly Pro Leu Leu Arg Ala Ile
                290                 295                 300

tgg cgc gtg ttc cgg tcc acc ttc ctg ctg ggg acc ctc agc ctg gtc      962
Trp Arg Val Phe Arg Ser Thr Phe Leu Leu Gly Thr Leu Ser Leu Val
            305                 310                 315

att agc gat gcc ttc agg ttt gct gtt ccc aag ctc ctc agt ctg ttt     1010
Ile Ser Asp Ala Phe Arg Phe Ala Val Pro Lys Leu Leu Ser Leu Phe
        320                 325                 330

ctg gag ttc atg ggt gac cgc aac tcc tcg gcg tgg aca ggc tgg ctc     1058
Leu Glu Phe Met Gly Asp Arg Asn Ser Ser Ala Trp Thr Gly Trp Leu
    335                 340                 345

cta gct gtg ctg atg ttc gcg gca gcc tgc cta cag acg ttg ttt gaa     1106
Leu Ala Val Leu Met Phe Ala Ala Ala Cys Leu Gln Thr Leu Phe Glu
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350                 355                 360                 365

cag cag cac atg tac aga gcc aag gtc ctg cag atg agg ctg cga aca     1154
Gln Gln His Met Tyr Arg Ala Lys Val Leu Gln Met Arg Leu Arg Thr
                370                 375                 380

gcc atc act ggc ctg gtg tac aga aag gtc ctg gtc ctg tcc agt ggt     1202
Ala Ile Thr Gly Leu Val Tyr Arg Lys Val Leu Val Leu Ser Ser Gly
            385                 390                 395

tcc aga aag tcc agc gca gca gga gac gtg gtc aac ctg gtg tcg gtg     1250
Ser Arg Lys Ser Ser Ala Ala Gly Asp Val Val Asn Leu Val Ser Val
        400                 405                 410

gac atc cag cgg ctg gcc gag agc atc atc tac ctc aac ggg ctg tgg     1298
Asp Ile Gln Arg Leu Ala Glu Ser Ile Ile Tyr Leu Asn Gly Leu Trp
    415                 420                 425

ctg ctc ttc ctg tgg atc ttt gtg tgc ttt gtc tac ctg tgg cag ctc     1346
Leu Leu Phe Leu Trp Ile Phe Val Cys Phe Val Tyr Leu Trp Gln Leu
430                 435                 440                 445

ctt gga ccc tct gct ctc aca gcc gtt gct gtc ttc ctg agc ctc ctc     1394
Leu Gly Pro Ser Ala Leu Thr Ala Val Ala Val Phe Leu Ser Leu Leu
                450                 455                 460

cct ctg aac ttc ttc atc acc aag aag agg ggc ttc cat cag gaa gaa     1442
Pro Leu Asn Phe Phe Ile Thr Lys Lys Arg Gly Phe His Gln Glu Glu
            465                 470                 475

cag atg agg cag aag gcc tcc aga gca cgg ctc acc agc tcc atg ctc     1490
Gln Met Arg Gln Lys Ala Ser Arg Ala Arg Leu Thr Ser Ser Met Leu
        480                 485                 490

aga act gtg aga acc atc aag tcc cac ggc tgg gag cat gcc ttc ctg     1538
Arg Thr Val Arg Thr Ile Lys Ser His Gly Trp Glu His Ala Phe Leu
    495                 500                 505

gag cga ctc ctt cac atc cgg ggc cag gag ctc agc gcc ctg aag acc     1586
Glu Arg Leu Leu His Ile Arg Gly Gln Glu Leu Ser Ala Leu Lys Thr
510                 515                 520                 525

tcc acc ctc ctc ttc tct gtg tct ctc gtg tcc ttc caa gtg tct aca     1634
Ser Thr Leu Leu Phe Ser Val Ser Leu Val Ser Phe Gln Val Ser Thr
                530                 535                 540

ttt ctg gtg gcg ctg gtc gtg ttt gct gtc cac acc ctg gtg gca gag     1682
Phe Leu Val Ala Leu Val Val Phe Ala Val His Thr Leu Val Ala Glu
            545                 550                 555

gac aat gcc atg gat gca gag aag gcc ttt gtg acg ctc aca gtg ctc     1730
Asp Asn Ala Met Asp Ala Glu Lys Ala Phe Val Thr Leu Thr Val Leu
        560                 565                 570

agc atc ctt aac aaa gcc cag gcc ttc ctc ccc ttc tct gtg cac tgc     1778
Ser Ile Leu Asn Lys Ala Gln Ala Phe Leu Pro Phe Ser Val His Cys
    575                 580                 585
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atc gtt cag gct cga gtg tcc ttt gac cgg ctg gct gcc ttc ctg tgc      1826
Ile Val Gln Ala Arg Val Ser Phe Asp Arg Leu Ala Ala Phe Leu Cys
590                 595                 600                 605

ctg gaa gaa gta gac ccc aat ggc atg atc gcg agt aac tcc agg cgc      1874
Leu Glu Glu Val Asp Pro Asn Gly Met Ile Ala Ser Asn Ser Arg Arg
                610                 615                 620

tcc tcg aag gat cga att tct gta cac aat ggc acc ttc gct tgg tcc      1922
Ser Ser Lys Asp Arg Ile Ser Val His Asn Gly Thr Phe Ala Trp Ser
            625                 630                 635

cag gag agc cca ccc tgc ctg cac ggg atc aac ctc acc gtg ccc cag      1970
Gln Glu Ser Pro Pro Cys Leu His Gly Ile Asn Leu Thr Val Pro Gln
        640                 645                 650

ggc tgt ctg ctg gct gtt gtg ggt cca gtg ggg gct ggg aag tcc tcc      2018
Gly Cys Leu Leu Ala Val Val Gly Pro Val Gly Ala Gly Lys Ser Ser
    655                 660                 665

ctg ctg tct gcc ctg ctt ggg gag ctg ttg aag gta gaa ggg tct gtg      2066
Leu Leu Ser Ala Leu Leu Gly Glu Leu Leu Lys Val Glu Gly Ser Val
670                 675                 680                 685

agc att gag ggt tcc gtg gct tac gtg cct cag gag gcc tgg gtc cag      2114
Ser Ile Glu Gly Ser Val Ala Tyr Val Pro Gln Glu Ala Trp Val Gln
                690                 695                 700

aat acc tct gtg gcg gag aat gtg tgc ttc agg caa gag ctg gac ctg      2162
Asn Thr Ser Val Ala Glu Asn Val Cys Phe Arg Gln Glu Leu Asp Leu
            705                 710                 715

ccc tgg ttg cag aaa gtt cta gac gcc tgt gcc ttg ggg tct gat gtg      2210
Pro Trp Leu Gln Lys Val Leu Asp Ala Cys Ala Leu Gly Ser Asp Val
        720                 725                 730

gcc agc ttc cct gca gga gtt cac acc cca ata ggg gag cag ggc atg      2258
Ala Ser Phe Pro Ala Gly Val His Thr Pro Ile Gly Glu Gln Gly Met
    735                 740                 745

aat ctt tct ggg ggc cag aag cag cgg ctg agc ttg gct cgg gct gtg      2306
Asn Leu Ser Gly Gly Gln Lys Gln Arg Leu Ser Leu Ala Arg Ala Val
750                 755                 760                 765

tac aaa aag gct gcc atc tac ttg ctg gat gac ccc ctg gca gcg ctg      2354
Tyr Lys Lys Ala Ala Ile Tyr Leu Leu Asp Asp Pro Leu Ala Ala Leu
                770                 775                 780

gat gcc cac gtc agc cag cag gtc ttc aaa cag gtc atc ggg ccc agt      2402
Asp Ala His Val Ser Gln Gln Val Phe Lys Gln Val Ile Gly Pro Ser
            785                 790                 795

gga ttg ctc cag ggt acg act cgg atc ctt gta aca cac acg ctg cac      2450
Gly Leu Leu Gln Gly Thr Thr Arg Ile Leu Val Thr His Thr Leu His
        800                 805                 810
```

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gtc ctg ccc cag gct gac cgg atc ctg gtg ctg gcc aat ggg acc atc      2498
Val Leu Pro Gln Ala Asp Arg Ile Leu Val Leu Ala Asn Gly Thr Ile
    815                 820                 825

gca gag atg ggc tcc tac cag gac ctt ctg caa agg aac gga gcc ctg      2546
Ala Glu Met Gly Ser Tyr Gln Asp Leu Leu Gln Arg Asn Gly Ala Leu
830                 835                 840                 845

gtg ggt ctt ctg gat gga gcc aga cag cct gca gga aca cac gat gca      2594
Val Gly Leu Leu Asp Gly Ala Arg Gln Pro Ala Gly Thr His Asp Ala
                850                 855                 860

gct acc agt gac gac ctc gga ggc ttt cct gga ggt ggg agg ccc aca      2642
Ala Thr Ser Asp Asp Leu Gly Gly Phe Pro Gly Gly Gly Arg Pro Thr
            865                 870                 875

tgc aga cca gac agg ccc agg ccc acg gag gca gcc cct gtg aag ggc      2690
Cys Arg Pro Asp Arg Pro Arg Pro Thr Glu Ala Ala Pro Val Lys Gly
        880                 885                 890

agg agc aca tct gag gta cag atg gag gct tct ctg gat gac cct gag      2738
Arg Ser Thr Ser Glu Val Gln Met Glu Ala Ser Leu Asp Asp Pro Glu
    895                 900                 905

gcc aca gga ttg aca gca gaa gag gat agt gtg cga tat ggc cgg gtg      2786
Ala Thr Gly Leu Thr Ala Glu Glu Asp Ser Val Arg Tyr Gly Arg Val
910                 915                 920                 925

aag atc acc ata tac ctg agc tac ctg cgg gcg gtg ggc aca ccc ctc      2834
Lys Ile Thr Ile Tyr Leu Ser Tyr Leu Arg Ala Val Gly Thr Pro Leu
                930                 935                 940

tgt acc tac acc ctg ttc ctc ttc ctc tgc cag caa gtg gca tcc ttc      2882
Cys Thr Tyr Thr Leu Phe Leu Phe Leu Cys Gln Gln Val Ala Ser Phe
            945                 950                 955

tcc caa ggc tac tgg ctg agc ctt tgg gcc gat gac ccg gtt gtg gat      2930
Ser Gln Gly Tyr Trp Leu Ser Leu Trp Ala Asp Asp Pro Val Val Asp
        960                 965                 970

ggg cgg cag atg cat gca gcc ctg cgt ggc tgg gtc ttt ggg ctc ctt      2978
Gly Arg Gln Met His Ala Ala Leu Arg Gly Trp Val Phe Gly Leu Leu
    975                 980                 985

ggc tgt ctg caa gcc atc gga ctg ttt gcc tcc  atg gct gcg gtg ttc     3026
Gly Cys Leu Gln Ala Ile Gly Leu Phe Ala Ser  Met Ala Ala Val Phe
990                 995                 1000                1005

ctg ggt gga gcc cgg  gcc tca ggc ctc ctt  ttc cgg agt ctc ctg       3071
Leu Gly Gly Ala Arg  Ala Ser Gly Leu Leu  Phe Arg Ser Leu Leu
                1010                1015                1020

tgg gac gtg gct cgc  tct ccc atc ggc ttc  ttt gag cgc acg cca       3116
Trp Asp Val Ala Arg  Ser Pro Ile Gly Phe  Phe Glu Arg Thr Pro
                1025                1030                1035

gtc ggg aac ctg ctg  aac cgc ttt tcc aag  gag aca gac aca gtg       3161
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Val Gly Asn Leu Leu  Asn Arg Phe Ser Lys  Glu Thr Asp Thr Val
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gat gtg gac atc ccg  gac aag ctg agg tcc  ctt ctg acc tat gcc      3206
Asp Val Asp Ile Pro  Asp Lys Leu Arg Ser  Leu Leu Thr Tyr Ala
                1055                1060                1065

ttt ggg ctc ctg gag  gtc ggc ctg gca gtg  acg atg gcc acg cct      3251
Phe Gly Leu Leu Glu  Val Gly Leu Ala Val  Thr Met Ala Thr Pro
                1070                1075                1080

ctg gcc att gtg gcc  atc cta cct ctc atg  gtc ctc tat gct ggg      3296
Leu Ala Ile Val Ala  Ile Leu Pro Leu Met  Val Leu Tyr Ala Gly
                1085                1090                1095

ttt cag agc ctc tat  gtg gcc aca tct tgc  cag ctg aga cgt cta      3341
Phe Gln Ser Leu Tyr  Val Ala Thr Ser Cys  Gln Leu Arg Arg Leu
                1100                1105                1110

gag tca gcc cgc tac  tca tct gtg tgt tcc  cat atg gct gag acc      3386
Glu Ser Ala Arg Tyr  Ser Ser Val Cys Ser  His Met Ala Glu Thr
                1115                1120                1125

ttc cag gga agt ctg  gtg gtc agg gcc ttc  cgg gcc cag gcg tcc      3431
Phe Gln Gly Ser Leu  Val Val Arg Ala Phe  Arg Ala Gln Ala Ser
                1130                1135                1140

ttc acg gct cag cac  gat gct ctc atg gat  gag aac cag agg gtc      3476
Phe Thr Ala Gln His  Asp Ala Leu Met Asp  Glu Asn Gln Arg Val
                1145                1150                1155

agt ttc ccg aaa ctg  gtg gct gac agg tgg  ctg gct act aac ctg      3521
Ser Phe Pro Lys Leu  Val Ala Asp Arg Trp  Leu Ala Thr Asn Leu
                1160                1165                1170

gag ctt cta ggg aat  ggc ttg gta ttc gtg  gct gct aca tgt gct      3566
Glu Leu Leu Gly Asn  Gly Leu Val Phe Val  Ala Ala Thr Cys Ala
                1175                1180                1185

gtg ctg agc aag gct  cac cta agt gct ggc  ctc gtg ggc ttc tcg      3611
Val Leu Ser Lys Ala  His Leu Ser Ala Gly  Leu Val Gly Phe Ser
                1190                1195                1200

gtc tcc gct gcc ctc  cag gtg aca cag act  ctg cag tgg gtg gtc      3656
Val Ser Ala Ala Leu  Gln Val Thr Gln Thr  Leu Gln Trp Val Val
                1205                1210                1215

cgc agc tgg aca gat  ctg gag aac agc atg  gta gcc gtg gag cgc      3701
Arg Ser Trp Thr Asp  Leu Glu Asn Ser Met  Val Ala Val Glu Arg
                1220                1225                1230

gtg cag gac tac gct  cgc atc ccc aaa gag  gct ccc tgg agg ctg      3746
Val Gln Asp Tyr Ala  Arg Ile Pro Lys Glu  Ala Pro Trp Arg Leu
                1235                1240                1245

ccc acc tgc gca gcc  cag cct ctc tgg cct  tgt ggg gga cag att      3791
Pro Thr Cys Ala Ala  Gln Pro Leu Trp Pro  Cys Gly Gly Gln Ile
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                1250                1255                1260

gag ttc cgg gac ttt  ggg ctc aga cac cga  cca gag ctg ccc ttg      3836
Glu Phe Arg Asp Phe  Gly Leu Arg His Arg  Pro Glu Leu Pro Leu
                1265                1270                1275

gct gtg cag gga gtg  tcc ctg aag atc cat  gca gga gag aag gtg      3881
Ala Val Gln Gly Val  Ser Leu Lys Ile His  Ala Gly Glu Lys Val
                1280                1285                1290

ggc atc gtg ggc aga  aca ggg gcc ggg aag  tcc tcc ctg gct tgg      3926
Gly Ile Val Gly Arg  Thr Gly Ala Gly Lys  Ser Ser Leu Ala Trp
                1295                1300                1305

ggc ctg ctg cgg ctt  cag gag gct gcc gag  ggt aat atc tgg atc      3971
Gly Leu Leu Arg Leu  Gln Glu Ala Ala Glu  Gly Asn Ile Trp Ile
                1310                1315                1320

gat ggg gtc cct atc  acc cat gtg ggg ctg  cac aca ctg agg tcc      4016
Asp Gly Val Pro Ile  Thr His Val Gly Leu  His Thr Leu Arg Ser
                1325                1330                1335

cga atc acc atc atc  cct cag gac cct gtc  ctg ttc cca ggc tct      4061
Arg Ile Thr Ile Ile  Pro Gln Asp Pro Val  Leu Phe Pro Gly Ser
                1340                1345                1350

ctg cgg atg aac ctg  gac ctg ctt cag gag  cac aca gat gaa ggc      4106
Leu Arg Met Asn Leu  Asp Leu Leu Gln Glu  His Thr Asp Glu Gly
                1355                1360                1365

atc tgg gca gcg ctg  gag aca gtg cag ctc  aag gcc ttc gtg acc      4151
Ile Trp Ala Ala Leu  Glu Thr Val Gln Leu  Lys Ala Phe Val Thr
                1370                1375                1380

agc ctg cct ggc cag  ctg caa tat gag tgt  gca ggc cag gga gat      4196
Ser Leu Pro Gly Gln  Leu Gln Tyr Glu Cys  Ala Gly Gln Gly Asp
                1385                1390                1395

gac ctg agc gtg ggt  cat aaa cag ctc ctg  tgc ctg gca cga gcc      4241
Asp Leu Ser Val Gly  His Lys Gln Leu Leu  Cys Leu Ala Arg Ala
                1400                1405                1410

ctt ctc cgg aaa acc  cag atc ctc atc ctg  gac gag gcg act gcc      4286
Leu Leu Arg Lys Thr  Gln Ile Leu Ile Leu  Asp Glu Ala Thr Ala
                1415                1420                1425

tct gtg gac cca ggg  acg gag atg cag atg  cag gcg gcc ctg gag      4331
Ser Val Asp Pro Gly  Thr Glu Met Gln Met  Gln Ala Ala Leu Glu
                1430                1435                1440

cgc tgg ttt aca cag  tgt acc tta ctg ctt  atc gct cac cgc ctg      4376
Arg Trp Phe Thr Gln  Cys Thr Leu Leu Leu  Ile Ala His Arg Leu
                1445                1450                1455

cgc tcc gtg atg gac  tgt gcc aga gtc cta  gtc atg gat gag ggg      4421
Arg Ser Val Met Asp  Cys Ala Arg Val Leu  Val Met Asp Glu Gly
                1460                1465                1470
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cag gtg gca gaa agt  ggc aat cct gct cag  ctg ctg gcc cag aaa       4466
Gln Val Ala Glu Ser  Gly Asn Pro Ala Gln  Leu Leu Ala Gln Lys
                1475                1480                1485

ggc ctg ttt tac agg  cta gcc cat gag tcg  ggc ctc gct tga          4508
Gly Leu Phe Tyr Arg  Leu Ala His Glu Ser  Gly Leu Ala
                1490                1495

atgaggattc ttaccaaccc ccgtggagcc agccatagag cctgcagtgg ctggagatgc   4568

cagagactcc aatctaaact cctctttggg agggagatgg cagagaaagt gatggagtat   4628

tgggatacca gacccagaag aacccagcac gcccaggttg gcctgagcaa ggccatgccc   4688

accccaggcc aaagagaatg gtaactctca gcccaagctg tctacttcaa ggccacgccc   4748

actccaggcc aatcagattg gatgccctgg acccaggtga tggtgtgcac atattcccta   4808

actccttatt ttgaagtcat tgtagatttc agtcacagtt ttaagaaata acacggagag   4868

aaactgtgac ccctctgccc tgtttattcc aagggtgaca ccttgtccaa ctctagagca   4928

tcacaccgac tctgaccgac tcgtctttac aactccaaaa aaaaaaaaaa aa            4980
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<210> 9
<211> 1498
<212> PRT
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<400> 9

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Met Asn Ser Gly Arg Ser Met Ala Thr Pro Gly Glu Gln Cys Ala Gly
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Leu Arg Val Trp Asn Gln Thr Glu Gln Glu Pro Ala Ala Tyr His Leu
            20                  25                  30

Leu Ser Leu Cys Phe Val Arg Ala Ala Ser Ser Trp Val Pro Pro Met
        35                  40                  45

Tyr Leu Trp Val Leu Gly Pro Ile Tyr Leu Leu Tyr Ile His Arg His
    50                  55                  60

Gly Arg Cys Tyr Leu Arg Met Ser His Leu Phe Lys Thr Lys Met Val
65                  70                  75                  80

Leu Gly Leu Ala Leu Ile Leu Leu Tyr Thr Phe Asn Val Ala Val Pro
                85                  90                  95
```

```
Leu Trp Arg Ile His Gln Gly Val Pro Gln Ala Pro Glu Leu Leu Ile
            100                 105                 110

His Pro Thr Val Trp Leu Thr Thr Met Ser Phe Ala Thr Phe Leu Ile
        115                 120                 125

His Met Glu Arg Arg Lys Gly Val Arg Ser Ser Gly Val Leu Phe Gly
    130                 135                 140

Tyr Trp Leu Leu Cys Cys Ile Leu Pro Gly Ile Asn Thr Val Gln Gln
145                 150                 155                 160

Ala Ser Ala Gly Asn Leu Arg Gln Glu Pro Leu His His Leu Ala Thr
                165                 170                 175

Tyr Leu Cys Leu Ser Leu Val Val Ala Glu Leu Val Leu Ser Cys Leu
            180                 185                 190

Val Asp Gln Pro Pro Phe Phe Ser Glu Asp Ser Gln Pro Leu Asn Pro
        195                 200                 205

Cys Pro Glu Ala Glu Ala Ser Phe Pro Ser Lys Ala Met Phe Trp Trp
    210                 215                 220

Ala Ser Gly Leu Leu Trp Arg Gly Tyr Lys Lys Leu Leu Gly Pro Lys
225                 230                 235                 240

Asp Leu Trp Ser Leu Gly Arg Glu Asn Ser Ser Glu Glu Leu Val Ser
                245                 250                 255

Gln Leu Glu Arg Glu Trp Arg Arg Ser Cys Asn Gly Leu Pro Gly His
            260                 265                 270

Lys Gly His Ser Ser Val Gly Ala Pro Glu Thr Glu Ala Phe Leu Gln
        275                 280                 285

Pro Glu Arg Ser Gln Arg Gly Pro Leu Leu Arg Ala Ile Trp Arg Val
    290                 295                 300

Phe Arg Ser Thr Phe Leu Leu Gly Thr Leu Ser Leu Val Ile Ser Asp
305                 310                 315                 320

Ala Phe Arg Phe Ala Val Pro Lys Leu Leu Ser Leu Phe Leu Glu Phe
```

325 330 335

Met Gly Asp Arg Asn Ser Ser Ala Trp Thr Gly Trp Leu Leu Ala Val
340 345 350

Leu Met Phe Ala Ala Ala Cys Leu Gln Thr Leu Phe Glu Gln Gln His
355 360 365

Met Tyr Arg Ala Lys Val Leu Gln Met Arg Leu Arg Thr Ala Ile Thr
370 375 380

Gly Leu Val Tyr Arg Lys Val Leu Val Leu Ser Ser Gly Ser Arg Lys
385 390 395 400

Ser Ser Ala Ala Gly Asp Val Val Asn Leu Val Ser Val Asp Ile Gln
405 410 415

Arg Leu Ala Glu Ser Ile Ile Tyr Leu Asn Gly Leu Trp Leu Leu Phe
420 425 430

Leu Trp Ile Phe Val Cys Phe Val Tyr Leu Trp Gln Leu Leu Gly Pro
435 440 445

Ser Ala Leu Thr Ala Val Ala Val Phe Leu Ser Leu Leu Pro Leu Asn
450 455 460

Phe Phe Ile Thr Lys Lys Arg Gly Phe His Gln Glu Glu Gln Met Arg
465 470 475 480

Gln Lys Ala Ser Arg Ala Arg Leu Thr Ser Ser Met Leu Arg Thr Val
485 490 495

Arg Thr Ile Lys Ser His Gly Trp Glu His Ala Phe Leu Glu Arg Leu
500 505 510

Leu His Ile Arg Gly Gln Glu Leu Ser Ala Leu Lys Thr Ser Thr Leu
515 520 525

Leu Phe Ser Val Ser Leu Val Ser Phe Gln Val Ser Thr Phe Leu Val
530 535 540

Ala Leu Val Val Phe Ala Val His Thr Leu Val Ala Glu Asp Asn Ala
545 550 555 560

```
Met Asp Ala Glu Lys Ala Phe Val Thr Leu Thr Val Leu Ser Ile Leu
                565                 570                 575

Asn Lys Ala Gln Ala Phe Leu Pro Phe Ser Val His Cys Ile Val Gln
            580                 585                 590

Ala Arg Val Ser Phe Asp Arg Leu Ala Ala Phe Leu Cys Leu Glu Glu
        595                 600                 605

Val Asp Pro Asn Gly Met Ile Ala Ser Asn Ser Arg Arg Ser Ser Lys
    610                 615                 620

Asp Arg Ile Ser Val His Asn Gly Thr Phe Ala Trp Ser Gln Glu Ser
625                 630                 635                 640

Pro Pro Cys Leu His Gly Ile Asn Leu Thr Val Pro Gln Gly Cys Leu
                645                 650                 655

Leu Ala Val Val Gly Pro Val Gly Ala Gly Lys Ser Ser Leu Leu Ser
            660                 665                 670

Ala Leu Leu Gly Glu Leu Leu Lys Val Glu Gly Ser Val Ser Ile Glu
        675                 680                 685

Gly Ser Val Ala Tyr Val Pro Gln Glu Ala Trp Val Gln Asn Thr Ser
    690                 695                 700

Val Ala Glu Asn Val Cys Phe Arg Gln Glu Leu Asp Leu Pro Trp Leu
705                 710                 715                 720

Gln Lys Val Leu Asp Ala Cys Ala Leu Gly Ser Asp Val Ala Ser Phe
                725                 730                 735

Pro Ala Gly Val His Thr Pro Ile Gly Glu Gln Gly Met Asn Leu Ser
            740                 745                 750

Gly Gly Gln Lys Gln Arg Leu Ser Leu Ala Arg Ala Val Tyr Lys Lys
        755                 760                 765

Ala Ala Ile Tyr Leu Leu Asp Asp Pro Leu Ala Ala Leu Asp Ala His
    770                 775                 780
```

```
Val Ser Gln Gln Val Phe Lys Gln Val Ile Gly Pro Ser Gly Leu Leu
785                 790                 795                 800

Gln Gly Thr Thr Arg Ile Leu Val Thr His Thr Leu His Val Leu Pro
                805                 810                 815

Gln Ala Asp Arg Ile Leu Val Leu Ala Asn Gly Thr Ile Ala Glu Met
            820                 825                 830

Gly Ser Tyr Gln Asp Leu Leu Gln Arg Asn Gly Ala Leu Val Gly Leu
        835                 840                 845

Leu Asp Gly Ala Arg Gln Pro Ala Gly Thr His Asp Ala Ala Thr Ser
    850                 855                 860

Asp Asp Leu Gly Gly Phe Pro Gly Gly Gly Arg Pro Thr Cys Arg Pro
865                 870                 875                 880

Asp Arg Pro Arg Pro Thr Glu Ala Ala Pro Val Lys Gly Arg Ser Thr
                885                 890                 895

Ser Glu Val Gln Met Glu Ala Ser Leu Asp Asp Pro Glu Ala Thr Gly
            900                 905                 910

Leu Thr Ala Glu Glu Asp Ser Val Arg Tyr Gly Arg Val Lys Ile Thr
        915                 920                 925

Ile Tyr Leu Ser Tyr Leu Arg Ala Val Gly Thr Pro Leu Cys Thr Tyr
    930                 935                 940

Thr Leu Phe Leu Phe Leu Cys Gln Gln Val Ala Ser Phe Ser Gln Gly
945                 950                 955                 960

Tyr Trp Leu Ser Leu Trp Ala Asp Asp Pro Val Val Asp Gly Arg Gln
                965                 970                 975

Met His Ala Ala Leu Arg Gly Trp Val Phe Gly Leu Leu Gly Cys Leu
            980                 985                 990

Gln Ala Ile Gly Leu Phe Ala Ser  Met Ala Ala Val Phe  Leu Gly Gly
        995                 1000                1005
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Ala Arg Ala Ser Gly Leu Leu Phe Arg Ser Leu Leu Trp Asp Val
1010 1015 1020

Ala Arg Ser Pro Ile Gly Phe Phe Glu Arg Thr Pro Val Gly Asn
1025 1030 1035

Leu Leu Asn Arg Phe Ser Lys Glu Thr Asp Thr Val Asp Val Asp
1040 1045 1050

Ile Pro Asp Lys Leu Arg Ser Leu Leu Thr Tyr Ala Phe Gly Leu
1055 1060 1065

Leu Glu Val Gly Leu Ala Val Thr Met Ala Thr Pro Leu Ala Ile
1070 1075 1080

Val Ala Ile Leu Pro Leu Met Val Leu Tyr Ala Gly Phe Gln Ser
1085 1090 1095

Leu Tyr Val Ala Thr Ser Cys Gln Leu Arg Arg Leu Glu Ser Ala
1100 1105 1110

Arg Tyr Ser Ser Val Cys Ser His Met Ala Glu Thr Phe Gln Gly
1115 1120 1125

Ser Leu Val Val Arg Ala Phe Arg Ala Gln Ala Ser Phe Thr Ala
1130 1135 1140

Gln His Asp Ala Leu Met Asp Glu Asn Gln Arg Val Ser Phe Pro
1145 1150 1155

Lys Leu Val Ala Asp Arg Trp Leu Ala Thr Asn Leu Glu Leu Leu
1160 1165 1170

Gly Asn Gly Leu Val Phe Val Ala Ala Thr Cys Ala Val Leu Ser
1175 1180 1185

Lys Ala His Leu Ser Ala Gly Leu Val Gly Phe Ser Val Ser Ala
1190 1195 1200

Ala Leu Gln Val Thr Gln Thr Leu Gln Trp Val Val Arg Ser Trp
1205 1210 1215

Thr Asp Leu Glu Asn Ser Met Val Ala Val Glu Arg Val Gln Asp

1220 1225 1230

Tyr Ala Arg Ile Pro Lys Glu Ala Pro Trp Arg Leu Pro Thr Cys
1235 1240 1245

Ala Ala Gln Pro Leu Trp Pro Cys Gly Gly Gln Ile Glu Phe Arg
1250 1255 1260

Asp Phe Gly Leu Arg His Arg Pro Glu Leu Pro Leu Ala Val Gln
1265 1270 1275

Gly Val Ser Leu Lys Ile His Ala Gly Glu Lys Val Gly Ile Val
1280 1285 1290

Gly Arg Thr Gly Ala Gly Lys Ser Ser Leu Ala Trp Gly Leu Leu
1295 1300 1305

Arg Leu Gln Glu Ala Ala Glu Gly Asn Ile Trp Ile Asp Gly Val
1310 1315 1320

Pro Ile Thr His Val Gly Leu His Thr Leu Arg Ser Arg Ile Thr
1325 1330 1335

Ile Ile Pro Gln Asp Pro Val Leu Phe Pro Gly Ser Leu Arg Met
1340 1345 1350

Asn Leu Asp Leu Leu Gln Glu His Thr Asp Glu Gly Ile Trp Ala
1355 1360 1365

Ala Leu Glu Thr Val Gln Leu Lys Ala Phe Val Thr Ser Leu Pro
1370 1375 1380

Gly Gln Leu Gln Tyr Glu Cys Ala Gly Gln Gly Asp Asp Leu Ser
1385 1390 1395

Val Gly His Lys Gln Leu Leu Cys Leu Ala Arg Ala Leu Leu Arg
1400 1405 1410

Lys Thr Gln Ile Leu Ile Leu Asp Glu Ala Thr Ala Ser Val Asp
1415 1420 1425

Pro Gly Thr Glu Met Gln Met Gln Ala Ala Leu Glu Arg Trp Phe
1430 1435 1440

Thr Gln Cys Thr Leu Leu Leu Ile Ala His Arg Leu Arg Ser Val
1445 1450 1455

Met Asp Cys Ala Arg Val Leu Val Met Asp Glu Gly Gln Val Ala
1460 1465 1470

Glu Ser Gly Asn Pro Ala Gln Leu Leu Ala Gln Lys Gly Leu Phe
1475 1480 1485

Tyr Arg Leu Ala His Glu Ser Gly Leu Ala
1490 1495

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<220>
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<400> 10
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<210> 11
<211> 20
<212> DNA
<213> Artificial

<220>
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<400> 11
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<210> 12
<211> 26
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<213> Artificial

<220>
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<210> 13
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<220>
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<210> 15
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<210> 16
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<400> 16
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<210> 17
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<212> DNA
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<400> 17
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<210> 18
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<220>
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<400> 18
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<210> 19
<211> 30
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<210> 20
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<210> 22
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<212> DNA
<213> Artificial

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<400> 22
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<210> 23
<211> 25
<212> DNA
<213> Artificial

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<400> 23
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<210> 24
<211> 25
<212> DNA
<213> Artificial

<220>
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<400> 24
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<210> 25
<211> 25
<212> DNA
<213> Artificial

<220>
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<400> 25
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<210> 26
<211> 21

<212> DNA
<213> Homo sapiens

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<400> 26
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Arg Ala Phe
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<210> 27
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> mutation in Intron 21 of human MRP6 gene

<400> 27
tacggcaggt taaccacc 18

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